Characterisation of the Murine Gammaherpesvirus-68 G Protein-Coupled Receptor

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Abstract

Gammaherpesviruses are lymphotropic viruses that establish lifelong latent infections in their host. The most intensively studied gammaherpesviruses are the clinically important Epstein Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV). EBV is associated with infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's lymphoma. KSHV is almost certainly the causative agent of Kaposi's sarcoma, body cavity based lymphoma and multicentric Castleman's disease. Analysis of EBV and KSHV is limited by the inherent species specificity of these viruses and their restricted growth *in vitro*. Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring virus found in wild rodents. It productively infects a range of cells and can be grown to high titres. MHV-68 also infects laboratory mice, thus making it an excellent small animal model for studying gammaherpesvirus infection. Herpesvirus genomes contain a variety of homologues of cellular genes involved in immune regulation and/or cell growth and proliferation. Cellular homologues encoded by MHV-68 include Bcl-2, cyclin D and a G protein-coupled receptor (GPCR) that shares greatest amino acid identity with the mammalian chemokine receptor, CXCR2. GPCRs are a superfamily of seven-transmembrane signalling molecules, and homologues occur in several herpesviruses. The KSHV GPCR is a functional, constitutively active oncoprotein that induces vascular endothelial growth factor.

The aim of this project was to characterise the MHV-68 GPCR (ORF74) and investigate its role in viral pathogenesis. This was approached in several ways. Firstly, the transcription pattern of the gene was determined using Northern analysis: GPCR expression was detected at early and late time-points during lytic infection on multiple rare transcripts. The size of the transcripts suggested they might be polycistronic and subsequent RT-PCR analysis demonstrated that the GPCR was co-expressed with v-Bcl-2, both *in vitro* and possibly *in vivo*. The subcellular localisation of the GPCR protein was investigated using an "epitope-tagging" strategy and immunofluorescence experiments revealed an expression pattern consistent with localisation to the cell surface. The transforming activity of the GPCR was examined using conventional tissue culture-based assays. NIH3T3 cells

expressing the GPCR exhibited focus formation and anchorage independent growth in soft agar. Lastly, a recombinant virus lacking the GPCR was generated by homologous recombination. However, this recombinant virus could not be purified from the parental wild type virus. In conclusion, the MHV-68 GPCR is a viral oncogene that is likely to contribute to the long-term persistence of MHV-68.

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Abbreviations

A	Adenosine
AHV-1	Alcelaphine herpesvirus 1
AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
AP	Alkaline phosphatase
Asn-X-Ser/Thr	Asparigine-X-Serine/Threonine
ATG	Initiating methionine
ATP	Adenosine triphosphate
dATP	Deoxyadenosine triphosphate
BART	*Bam*HIA rightward transcripts
BCBL	Body cavity based lymphoma
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	Baby hamster kidney
BL	Burkitt's lymphoma
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C9	2'-deoxy-5- ethyl-beta-4'thiouridine
cDNA	Complementary DNA
CIP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
dCTP	Deoxycytidine triphosphate
°C	Degrees Celsius
dH_2O	Distilled water
dNTP	Deoxynucleoside triphosphate
DMEM	Dulbecco's modified eagle's medium
DRY	Aspartic acid-arginine-tyrosine
DTT	Dithiothreitol
EBER	Epstein-Barr virus-encoded small RNA
EBNA	Epstein Barr virus nuclear antigen

EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	Ethylene diaminetetraacetic acid
EHV-2	Equine herpesvirus 2
FCS	Foetal calf serum
FITC	Fluorescine isothiocyanate
FLICE	FADD homologous ICE/CED-3-like protease
FLIP	FLICE- inhibitory protein
fMLP	*N*-formyl-L-methionyl-L-phenylalanine
GPCR	G protein-coupled receptor
GMCSF	Granulocyte macrophage colony stimulating factor
GMEM	Glasgow's modified Eagle's medium
GRO-α	Growth-related protein α
GST	Glutathione S-transferase
dGTP	Deoxyguanosine triphosphate
HA	Haemagglutinin
HCl	Hydrogen chloride
HEPES	N'-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HHV	Human herpesvirus
HSV	Herpes simplex virus
HVS	Herpesvirus saimiri
IF	Immunofluorescence
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IM	Infectious mononucleosis
IP-10	Interferon-induced protein-10
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRES	Internal ribosome entry site
I-TAC	Interferon-inducible T cell α attractant
kb	Kilobase
KS	Kaposi's sarcoma

KSHV	Kaposi's sarcoma associated herpesvirus
LANA	Latency associated nuclear antigen
LB	Luria Bertani medium
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LP	Leader protein
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester
MCD	Multicentric Castleman's disease
MCP	Monocyte chemoattractant protein
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MHV	Murine gammaherpesvirus
MIG	Monokine induced by interferon γ
MIP	Macrophage inhibitory protein
MOI	Multiplicity of Infection
MoMuLV	Moloney murine leukaemia virus
MOPS	Morpholinepropanesulphonic acid
mRNA	Messenger RNA
NAP	Neutrophil activating peptide
NBT	Nitro blue tetrazolium
NK	Natural killer
NPC	Nasopharyngeal carcinoma
NZW	New Zealand white
OD	Optical density
OHV	Ovine herpesvirus
Oligo(dT)	Oligodeoxythymidine
ORF	Open reading frame
ori	origin of replication
p	Prefix for plasmid DNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PEC	Peritoneal exudate cell
PEL	Primary effusion lymphoma
p.i.	Post-infection
pfu	Plaque forming unit
PMSF	Phenylmethylsulphonyl fluoride
RACE	Rapid amplification of cDNA ends
RANTES	Regulated upon activation, normal T-cell expressed & secreted
RCV	Recombinant virus
RNA	Ribonucleic acid
RPMI	Rosewell park memorial institute
RRV	Rhesus monkey rhadinovirus
RT	Room temperature
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SV40	Simian vacuolating virus 40
T	Thymine
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEMED	N, N, N', N'-tetraethylmethylenediamine
TPA	12-O-tetradecanoyl phorbol-13 acetate
Tris-HCl	Tris Hydrochloride
TTP	Thymidine triphosphate
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling
UV	Ultraviolet (light)
v/v	Volume per volume
VZV	Varicella zoster virus
WT	Wild type
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter One: Introduction

1.1. The Herpesviridae

The herpesviridae comprise a large family of viruses, numbering at least one hundred members that are widespread in both animals and humans. All herpesviruses share the ability to establish latent infection, which allows them to persist for the lifetime of the host. Eight human herpesviruses have been identified, several of which are endemic in the population. For instance, 95% of people carry the herpes simplex virus (HSV-1) and about 90% are seropositive for the Epstein Barr virus (EBV). In the majority of cases, reactivation or recrudescence does not occur in immunocompetent hosts. The genetic and environmental cues that elicit reactivation in some individuals but not others have not been clearly defined. However, herpesvirus reactivation is strongly associated with immunosuppression and, in this context, may cause serious and potentially fatal disease.

HSV-1 (HHV-1) and HSV-2 (HHV-2) recurrently infect epidermal tissue resulting in the development of cold sores and genital lesions, while varicella zoster virus (VZV, HHV-3) is the causative agent of chicken pox and shingles. These infections are usually resolved by the host's immune system but life-threatening complications such as meningitis and encephalitis can occur (Arvin, 1996; Whitley, 1996). EBV (HHV-4) is the aetiologic agent associated with infectious mononucleosis and a number of malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disease (Kieff, 1996). Cytomegalovirus (HCMV, HHV-5) infection is largely asymptomatic but vertical transmission can cause blindness, deafness or mental retardation in the developing foetus. A lethal disseminated CMV infection has also been reported in AIDS patients and transplant recipients receiving immunosuppressive drugs (Britt, 1996). CMV has also been associated with certain types of vascular disease such as atherosclerosis (Melnick *et al.*, 1996). Human herpesvirus 6 (HHV-6) is associated with the childhood disease, *Roseola infantum* and a link with multiple sclerosis has also been postulated. Little is known of any pathology associated with human herpesvirus 7 (HHV-7). Kaposi's sarcoma herpesvirus (KSHV, HHV-8) is a more recent addition to the family (Chang *et al.*, 1994). It is associated with Kaposi's sarcoma, body cavity based lymphoma

and multicentric Castleman's disease, a previously rare group of conditions now observed more commonly in AIDS patients.

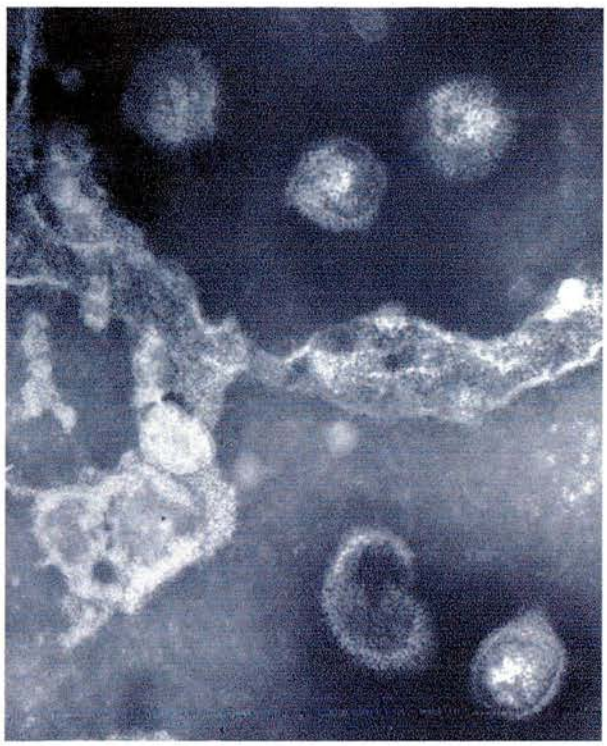
1.1.1. Herpesvirus Composition and Classification

Herpesviruses have large linear, double-stranded DNA genomes encased in icosahedral capsids that are 100-110nm in diameter and comprise 162 capsomeres (figure 1.1.1). Between the capsid and the envelope lies the tegument, an amorphous material consisting of virus proteins. The capsid is enveloped in host-cell derived membrane containing numerous virus glycoproteins that protrude from the membrane surface like spikes. Herpes simplex (HSV-1) possesses at least eleven different glycoproteins, some of which facilitate virus entry.

The size of the viral genome varies between 120-230kb. A size difference of up to 10kb has been observed between individual virions of the same virus strain that arises from the variability of the number of internal and terminal repeats. The herpesviruses have been classified into six subtypes (A-F) depending on the arrangement of the repeat structures and unique regions. The GC content of herpesvirus genomes ranges from 31-75% between family members and there is also heterogeneity in base sequence distribution across the length of the genome (Roizman, 1996b). Herpesvirus genomes are economically arranged: open reading frames tend to be closely spaced and often overlap; genes may be transcribed in leftward or rightward direction and use alternative initiation, polyadenylation or splice sites. The unique region of the genome encodes blocks of conserved genes that are present in the majority of herpesviruses (Chee & Barrell, 1990; Davison & Taylor, 1987). The conserved gene blocks are interspersed with genes that are unique to individual herpesviruses, although they may be encoded by more than one virus. The unique genes include a number of novel genes and also genes that are likely to have been acquired from the host cell genome (discussed in later sections).

The known herpesviruses appear to share four significant biological properties: they all synthesise a large number of enzymes involved in nucleic acid metabolism

Figure 1.1.1. Electron Micrograph of Epstein Barr Virions



Electron micrograph of un-enveloped Epstein Barr virions taken from a cell spread of EBV-infected cells. The image depicts the icosahedral structure of the herpesvirus nucleocapsid. Note the collapsed nucleocapsid towards the bottom right-hand corner. Adapted from (Madeley, 1972).

(e.g. thymidine kinase) and DNA replication (e.g. DNA polymerase); DNA synthesis and assembly of virus capsids occurs in the nucleus; lytic replication and production of infectious progeny results in destruction of the infected host cell; all herpesviruses have the capacity to establish a latent infection in natural host cells, with genomes adopting a closed circular formation and expressing a limited subset of genes. However, despite these similarities, members of the family can be differentiated by their specific biological properties. The herpesviruses have been clustered into three subfamilies (α, β, γ) on the basis of host range, duration of reproductive cycle, cytopathology, genome structure, characteristics of latent infection and more recently, genetic conservation (Roizman, 1982). Table 1.1.1 lists examples of each subfamily.

1.1.2. The Alphaherpesviruses

The alphaherpesvirinae are characterised by their variable host range, short reproductive cycle and rapid destruction of susceptible cells. Examples include HSV-1 and HSV-2 and VZV. The alphaherpesviruses establish latency primarily but not exclusively in the sensory ganglia.

1.1.3. The Betaherpesviruses

The betaherpesviruses have a comparatively long reproductive cycle and are less cytopathic, giving rise to typically enlarged cells (cytomegalia) exemplified by cytomegalovirus (CMV). Latency is established in a variety of tissues including secretory glands, lymphoreticular cells and kidneys. The human herpesviruses, HHV-6 and HHV-7, have recently been classified as betaherpesviruses (Gompels *et al.*, 1995; Nicholas, 1996). HHV-6 and HHV-7 are $CD4^+$ T cell tropic whereas CMV infects monocytes and lymphocytes.

1.1.4. The Gammaherpesviruses

The gammaherpesviruses such as EBV, KSHV and MHV-68 have a narrower host range than the alphaherpesviruses. All members replicate in lymphoblastoid cell lines and some can lytically infect fibroblastic and epithelial lines *in vitro*. Latency is established primarily in lymphocytes: EBV, KSHV and MHV-68 are B-cell

Table 1.1.1. Examples of the Herpesvirus Family

Name	Host	Disease
Alphaherpesviruses (α1 *Simplexvirus* and α2 *Varicellovirus* subgroups)		
Herpes simplex 1 (HSV-1)	Human	Cold sores, keratitis, ocular disease
Herpes simplex 2 (HSV-2)	Human	Genital herpes
Bovine herpesvirus 2 (BHV-2)	Cow	
Marek's Disease Virus (MDV)	Chicken	T cell lymphoma
Varicella zoster (VZV)	Human	Chicken pox, shingles
Pseudorabies virus	Pig	Aujeszky's disease
Equine herpesvirus 1 (EHV-1)	Horse	Spontaneous abortion
Betaherpesviruses (β1 *Cytomegalovirus* and β2 *Roseolovirus* subgroups)		
Cytomegalovirus (HCMV)	Human	Foetal deformities Lymphoproliferation in immunocompromised
Murine cytomegalovirus (MCMV)	Mouse	
Human herpesvirus 6 (HHV-6)	Human	*Roseola infantum*
Human herpesvirus 7 (HHV-7)	Human	
Gammaherpesviruses (γ1 *Lymphocryptovirus* and γ2 *Rhadinovirus* subgroups)		
Epstein Barr virus (EBV)	Human	Infectious mononucleosis, Burkitt's lymphoma Nasopharyngeal carcinoma Hodgkin's lymphoma
Herpesvirus pan	Chimpanzee	
Herpesvirus papio	Baboon	
Herpesvirus saimiri (HVS)	Squirrel Monkey	Fatal lymphoproliferative disease in cotton tail rabbits, New World monkeys e.g. marmosets
Kaposi's sarcoma herpesvirus (KSHV)	Human	Kaposi's sarcoma Body cavity based lymphoma Multicentric Castleman's disease
Herpesvirus aeteles	Spider Monkey	
Rhesus monkey rhadinovirus (RRV)	Old world monkey	Experimental lymphadenopathy and vascular hyperplasia
Equine herpesvirus 2 (EHV-2)	Horse	
Herpesvirus sylvilagus	Cotton tail rabbit	Lymphomas Lymphoproliferations
Woodchuck herpesvirus	Woodchuck	
Murine gammaherpesvirus 68 (MHV-68)	Murid rodents	Lymphomas
Bovine herpesvirus 4 (BHV-4)	Cow	
Alcelaphine herpesvirus 1	Wildebeest	Malignant catarrhal fever in cattle
Ovine herpesvirus 2 (OHV-2)	Sheep	Malignant catarrhal fever in cattle

Gammaherpesviruses are over-represented among these examples of herpesviruses as a gammaherpesvirus, MHV-68, is the focus of this thesis

tropic, whereas HVS is a T-cell tropic virus. Classification of the gammaherpesviruses into subgroups has proved difficult as members of the subfamily sharing cell tropism or other biological properties do not necessarily have similar genomic structure or sequence homology. However, two genera are recognised, *Lymphocryptovirus* (gamma-1-herpesvirus) and *Rhadinovirus* (gamma-2-herpesvirus), that are exemplified by EBV and HVS respectively.

1.1.5. Replication of Herpesviruses

Herpesvirus infection of a cell can result in two different replicative outcomes: lytic replication with production of virus progeny and consequent death of the host cell; or latent infection, during which the virus genome is maintained as a circular episome and expresses a restricted subset of genes. Replication of the latent genome occurs during host cell division and utilises host cell machinery but does not generate virus progeny. The term "lytic replication" implies cytolytic destruction of the cell, although not all herpesviruses engender cytolysis. A more accurate definition of herpesvirus replication that results in generation of progeny is "productive replication". However, herpesvirus terminology has been defined by the prototypical cytolytic herpesvirus, HSV-1, and the terms are used interchangeably.

Figure 1.1.5 depicts the lytic replication cycle schematically. In order to initiate infection, the virus must attach to receptors on the cell surface via an interaction with virion envelope glycoproteins. The cellular receptors utilised by herpesviruses vary between individual members of the family, as do the viral glycoproteins necessary for entry. A great deal of information about attachment, and indeed the lytic replication cycle, has been amassed from the study of HSV-1 and the generation of HSV-1 mutants. HSV-1 appears to require multiple glycoproteins for entry and uses more than one attachment pathway. The cell surface receptor is most likely the ubiquitous heparan sulphate, although HSV-1 is thought to interact with more than one cellular receptor. Conversely, the *in vitro* host range restriction of EBV to B lymphocytes is partly due to the specificity of the EBV glycoprotein, gp340/220, for the B-lineage-associated C3d complement receptor, CD21 (CR2).

Figure 1.1.5. Lytic Replication of the Epstein Barr Virus

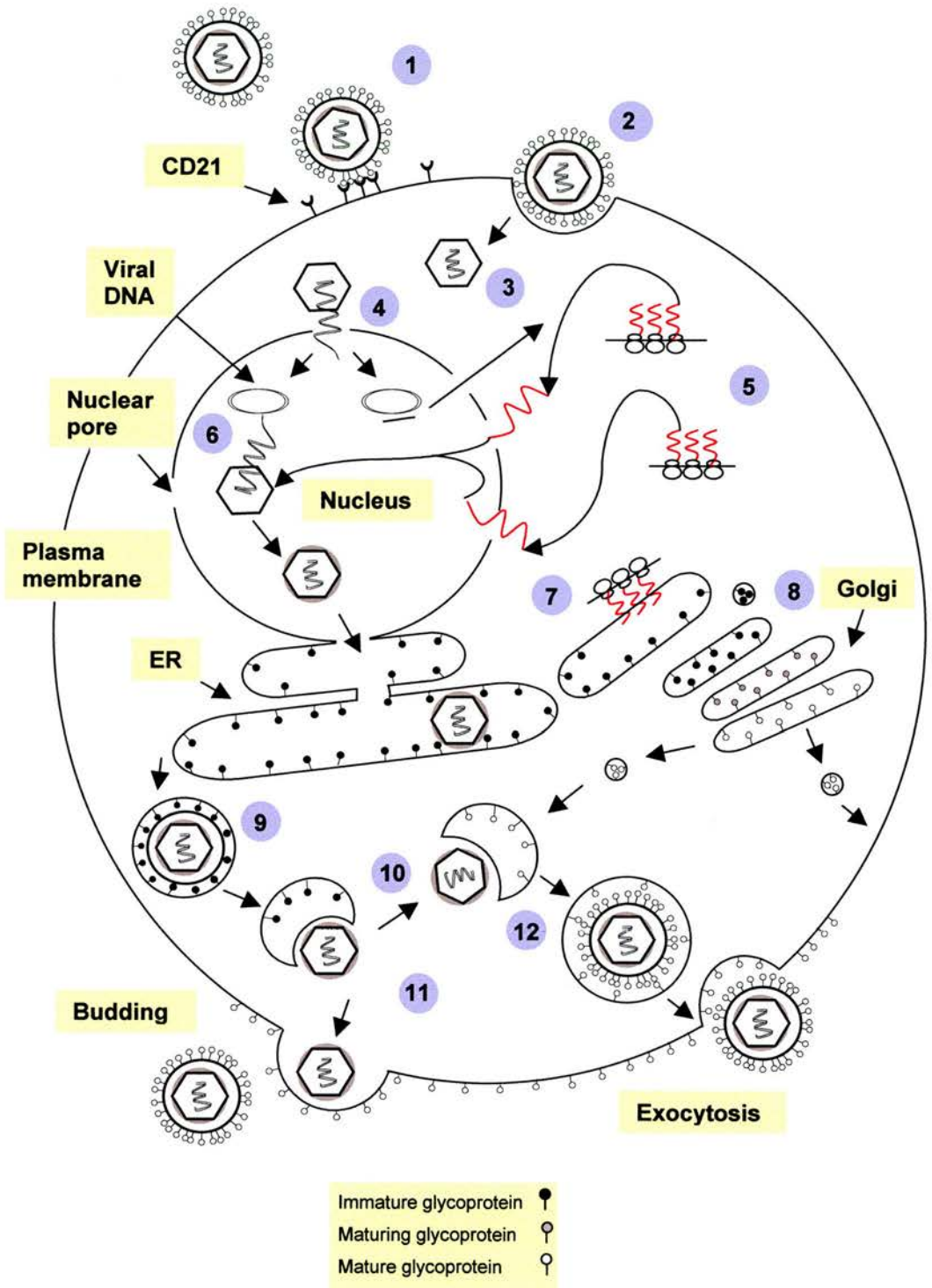


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Figure 1.1.5. 1. The virus attaches to the cell via an interaction between a virion membrane glycoprotein and a cell surface receptor, which in the case of EBV is the complement receptor CR2/CD21. 2. Following attachment, the virion is internalised into a vesicle derived from the plasma membrane. The virion membrane fuses with the vesicle membrane in a pH-independent manner, resulting in the release of the viral capsid into the cytoplasm. 3. The uncoated capsid traffics to the nuclear membrane and releases the linear double-stranded DNA virus genome into the nucleus. 4. The linear virus genome immediately circularises. 5. Sequential viral protein expression ensues; commencing with the immediate early proteins that include the viral transactivators required for expression of the early proteins such as DNA polymerase and other enzymes, which mediate DNA replication. Lastly, the late viral proteins are synthesised; these include the structural capsid proteins and viral glycoproteins. 6. Virus DNA replication occurs via a rolling circle mechanism: the concatenated genomes are cleaved and packaged into the newly formed nucleocapsids, which then enter the endoplasmic reticulum (ER). 7. Newly synthesised virus proteins are directed from the ribosome to the ER for modifications such as N-linked glycosylation. 8. The majority of glycoproteins also receive further processing in the Golgi before trafficking to the plasma membrane. 9. Vesicles release nucleocapsids into the cytoplasm. 10. It is proposed that the nucleocapsids acquire a definitive envelope either by 11. budding through the plasma membrane containing mature Golgi-processed glycoproteins or 12. by budding into post-Golgi derived vesicles containing viral glycoproteins prior to exocytosis of the enveloped virion. Adapted from (Roizman, 1996a) and (Gong & Kieff, 1990).

Following attachment, the HSV-1 envelope fuses directly with the plasma membrane resulting in endocytosis of the nucleocapsid, a process that is also dependent upon multiple viral glycoproteins. In the case of EBV, the virion is internalised and the virus envelope fuses with the vesicular membrane, releasing the nucleocapsid into the cytoplasm. Transport of nucleocapsids to the nucleus is probably mediated by the cell cytoskeleton (Kristensson *et al.*, 1986) and results in the release of viral DNA into the nucleus, where it immediately circularises. Several tegument proteins are important for the initiation of DNA replication such as the HSV-1 VP16 protein, a transactivator that induces transcription of the immediate early viral genes.

Transcription of viral DNA by host RNA polymerase II occurs in the nucleus in a co-ordinately regulated cascade. There are three kinetic groups of genes: the immediate early (α) genes, the early (β) genes and the late (γ) genes. In addition to these, herpesviruses encode a small subset of genes and non-translated RNAs during latent infection. There are five immediate early viral proteins, which are expressed in the absence of protein synthesis and all serve to regulate the sequential expression of the early and late gene subsets. In the main, the early genes encode the viral proteins necessary for nucleic acid metabolism, DNA replication and virus assembly e.g. DNA polymerase, helicase-primase, dUTPase and thymidine kinase; the virus structural proteins are encoded by the late genes.

The virus genome is replicated by a rolling circle mechanism, producing head-to-tail concatemers (Jacob & Roizman, 1977) that are cleaved at consensus sequences into single genome units and packaged into newly synthesised capsids (Deiss *et al.*, 1986). Late gene transactivators are expressed at early time points resulting in subsequent transcription of the late genes following DNA replication e.g. tegument proteins, glycoproteins.

Envelopment and egress of the virus from the cell is less well characterised. There is evidence to suggest that HSV-1 capsids bud through the nuclear membrane, acquiring immature viral glycoproteins that were transported there previously (Vlazny *et al.*, 1982). Immature virus particles would then pass through the Golgi

network, facilitating glycoprotein processing, and traffic to the plasma membrane in a Golgi-derived vesicle before being released from the cell by a process of reverse exocytosis (Desai *et al.*, 1988; Johnson & Spear, 1982). Alternatively, the nucleocapsid may acquire its final envelope at the plasma membrane. There is also evidence to suggest that the virus is enveloped in the Golgi and undergoes uncoating and re-envelopment steps during egress (Whiteley *et al.*, 1999). EBV envelopment is thought to occur at the plasma membrane or following envelopment by post-Golgi vesicles (Gong & Kieff, 1990).

1.2-1.5. The Gammaherpesviruses

The most intensively investigated gammaherpesvirus is EBV, although much interest is currently being directed towards KSHV. Of the non-human gammaherpesviruses, herpesvirus saimiri (HVS) and murine gammaherpesvirus 68 (MHV-68) are studied in the context of animal models for human disease. Gammaherpesviruses affecting cattle and other ruminants such as alcelaphine herpesvirus 1 (AHV-1) and ovine herpesvirus 2 (OHV-2) are also of considerable interest due to their economic impact on agriculture and devastating effect on conservation programmes.

1.2. The Epstein Barr Virus

EBV, the prototypical *Lymphocryptovirus*, is widespread in all human populations with approximately 90% of adults testing positively for EBV-specific antibody (Rowe *et al.*, 1988). Like all human herpesviruses, it establishes lifelong persistence in the host. Epstein and colleagues first isolated the virus from lymphoblastoid cell lines derived from an endemic Burkitt's lymphoma biopsy (Epstein, 1964). EBV is the aetiological agent associated with a number of human diseases. Primary infection with EBV, while often asymptomatic, can produce a condition known as infectious mononucleosis or glandular fever (Schooley, 1985). EBV is also the aetiologic agent associated with Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and post-transplant lymphoproliferative disease (BLPD). It has also been implicated in the pathogenesis of X-linked lymphoproliferative syndrome, Hodgkin's disease (Anagnostopoulos *et al.*, 1989), Sjogren's syndrome (Inoue *et al.*,

1991), rheumatoid arthritis (Moss *et al.*, 1983) and other epithelial malignancies such as carcinoma of the parotid gland (Jones *et al.*, 1988; Raab-Traub *et al.*, 1991).

The EBV genome is 172kb in length and comprises a short and a long unique region interspersed by 3kb internal repeats and flanked by 500bp terminal repeats. The EBV genome, which has a 60% GC content, encodes over 90 proteins and was the first herpesvirus to be fully cloned and sequenced (Baer *et al.*, 1984; Dambaugh *et al.*, 1980; Hatfull *et al.*, 1988). There are two subtypes, EBV-1 and EBV-2, which share extensive homology but exhibit variation in the small polyadenylated RNAs (EBERs) and the nuclear antigen genes (Sample *et al.*, 1990). EBV-1 is more prevalent in developed societies whereas African isolates show a more even distribution between EBV-1 and EBV-2 (Zimber *et al.*, 1986).

EBV, like other gammaherpesviruses, has a restricted host range with humans as its natural host. This presents difficulties when attempting to study the course of infection *in vivo* (see section 1.5.1 entitled MHV-68 as an animal model). EBV lytically infects squamous epithelial cells but the infection is far from efficient and no other fully permissive tissue culture system is available for studying productive infection. The virus infects and immortalises B lymphocytes *in vitro* (Pattengale *et al.*, 1973), generating lymphoblastoid cell lines which have been used to study EBV latent infection in depth (Pope *et al.*, 1968). In these cells, the genome immediately circularises upon infection and is maintained as an episome in the absence of productive replication. However, within an LCL population, a small number of cells (0-5%) undergo productive replication. The switch from latency to the lytic cycle is associated with B cell activation and differentiation (Crawford & Ando, 1986) and can be triggered using activating agents such as phorbol esters (zur Hausen *et al.*, 1978), sodium butyrate (Luka *et al.*, 1979) and anti-immunoglobulin (Tovey *et al.*, 1978).

1.2.1. Infectious Mononucleosis

Infectious mononucleosis (IM) is characterised by the presence of heterophile antibodies in the serum accompanied by an atypical lymphocytosis. Symptoms

include pharyngitis, lymphadenopathy, general malaise, fever and splenomegaly (Henle, 1979). However, the severity of the illness can vary from a mild fever to prolonged debilitating illness. Symptomatic IM is more likely to occur when the individual is infected in early adulthood, with 50% of these cases manifesting as IM. The virus replicates in the oropharyngeal epithelium and is intermittently secreted in the saliva of most seropositive individuals, thus resulting in oral transmission (Rickinson *et al.*, 1975; Yao *et al.*, 1989). While the primary site of lytic replication is the pharyngeal epithelium, the virus establishes latency in B lymphocytes. This is supported by the finding that B cell-deficient patients with X-linked agammaglobulinaemia are not infected with EBV (Faulkner et al., 1999). It is thought that recirculating B cells become infected in the oropharynx and that the bone marrow, lymphoid tissue and peripheral blood lymphocytes are major reservoirs for EBV (Decker *et al.*, 1996; Khan *et al.*, 1996). Approximately one in one million B cells of asymptomatic carriers are latently infected with EBV (Khan *et al.*, 1996; Lewin *et al.*, 1987; Miyashita *et al.*, 1995). However, sporadic reactivation of the virus and proliferation of EBV-infected cells is held in check by virus-specific cytotoxic T cells (Moss *et al.*, 1979; Rickinson *et al.*, 1979; Rickinson *et al.*, 1980). The importance of this immune surveillance is highlighted in the case of immunosuppressed transplant patients who frequently experience lymphoproliferation of EBV-infected B cells, resulting in malignant lymphomas (Hanto *et al.*, 1983).

1.2.2. Burkitt's Lymphoma

Burkitt's lymphoma occurs in three recognised forms: endemic, sporadic and AIDS-associated BL. Endemic BL was first described by Denis Burkitt, a surgeon working in Africa, and is most tightly associated with EBV (Burkitt, 1963). BL, a B cell tumour localised to the jaw, is the most common childhood lymphoma in equatorial Africa and Papua New Guinea and affects approximately 1 in 10,000 individuals annually. Without treatment, BL is fatal within a few months.

The EBV genome is detectable in the vast majority of tumours (97%, zur Hausen & Schulte-Holthausen, 1970) and patients exhibit high levels of virus-specific

antibodies about five years prior to the onset of clinical symptoms (Geser *et al.*, 1982). EBV is considered to be a necessary element in the causation of BL, although other risk factors such as hyperendemic falciparum malaria, or HIV in the case of AIDS-associated BL, act together in a complex chain of events leading to malignant change. BL cells possess a characteristic chromosomal translocation, t(8:14, 2 or 22), resulting in juxtaposition of the c-*myc* oncogene and the Ig heavy-chain locus (chromosome 14, 80% of tumours) or one of the light-chain loci. It is thought that infection with malaria leads to a high level of EBV-driven B cell proliferation that increases the probability of a translocation (Klein, 1985). Subsequent deregulation of c-*myc* results in B cell proliferation and hence BL (Bornkamm *et al.*, 1987; Klein, 1985; Leder *et al.*, 1983).

1.2.3. Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a squamous epithelial tumour localised to the postnasal space. It is especially prevalent in southern China where it is the most common cancer affecting men and the second commonest cancer of women. A genetic predisposition has been demonstrated in this population (Chan, 1983; Simons *et al.*, 1974) but studies on Chinese migrants have implicated environmental co-factors such as traditional Chinese herbal remedies and salt fish dishes, which contain tumour-promoting phorbol esters and nitrosamines respectively (Buell, 1974; Hirayama & Ito, 1981; Huang, 1978). An association of EBV with NPC was proposed following the observation that patients exhibit elevated levels of EBV-specific antibodies (Old, 1966) and that the EBV genome can be detected in all NPC samples (Desgranges *et al.*, 1982; Nonoyama & Pagano, 1973; Wolf *et al.*, 1973).

1.2.4. EBV and Immunosuppression

The occurrence of EBV-driven lymphomas has been described in immunosuppressed individuals (Crawford *et al.*, 1980; Kalter *et al.*, 1985). In these cases, loss of immune surveillance results in increased viral replication in the oropharynx and B-cell proliferation. Again, detection of EBV-specific antigens in these tumours suggests a strong association with the virus (Herndier *et al.*, 1994; Thomas *et al.*, 1990). Organ graft recipients, who receive lifelong immunosuppressive drugs that

inhibit T cell activity, are 28-100 times more likely to develop lymphoproliferative disease (Penn, 1983). Reducing the level of immunosuppressive drugs has been shown to cause regression but carries the risk of graft rejection (Starzl *et al.*, 1984). It may be possible to control the condition by adoptive transfer of EBV-specific cytotoxic T cells (Haque *et al.*, 1998; Rooney *et al.*, 1995). In addition to BL, AIDS patients frequently develop large-cell lymphomas and oral hairy leukoplakia (Greenspan *et al.*, 1985). The vital role of the immune system in controlling proliferation of EBV-infected B cells is also demonstrated in cases of X-linked immunodeficiency. Following primary infection with EBV, 75% of affected men succumb to fatal IM. Those who survive are at risk of developing hypogammaglobuliaemia or lymphoma (Purtilo *et al.*, 1992; Tatsumi & Purtilo, 1986).

1.2.5. Other EBV-Associated Diseases

EBV genomes and viral proteins are present in approximately 60% of cases of Hodgkin's disease and there is an epidemiological correlation between IM in early adulthood and the development of Hodgkin's disease in later life (Herbst *et al.*, 1991; Weiss *et al.*, 1989). EBV has also been detected, in a varying percentage of cases, in certain types of T cell lymphoma: angioimmunoblastic lymphadenopathy (Ott *et al.*, 1992); nasal lymphoma (Harabuchi *et al.*, 1990) and peripheral T cell lymphoma (Jones *et al.*, 1988). In all these instances, the contribution of EBV to pathogenesis remains unclear.

1.2.6. EBV Latent Gene Expression

Like all herpesviruses, EBV expresses a highly restricted subset of genes during latent infection. The latency-associated genes encoded by EBV are unique genes and no homologues have been found in other herpesviruses (Virgin *et al.*, 1997). Several distinct patterns of gene expression have been observed in different *in vitro* and *in vivo* circumstances and have been assigned to three groups: latency type I, II and III.

The full array of latent genes is expressed in latency type III: six EBV nuclear antigens (EBNAs), EBNA-1, 2, 3A, 3B, 3C and LP (leader protein); three latent

membrane proteins (LMP), LMP-1, 2A and 2B; two EBV infected cell RNAs known as EBERs and a group of un-characterised transcripts designated as the BARTs to denote the region of the genome they originate from (Brooks *et al.*, 1993). This pattern of expression is observed in LCLs, infectious mononucleosis and post-transplant lymphomas.

EBNA-1 is a DNA-binding protein that binds to specific sequences in the latent viral origin of replication (*oriP*) and is required for maintenance of the viral episome. It also transactivates expression of the other latency genes and has a role in tethering the virus genome to host chromosomes thus ensuring equal partitioning during cell division. EBNA-1 contains a glycine-alanine repeat sequence, which enables it to escape degradation by the proteasome and therefore antigen presentation to cytotoxic T cells (Levitskaya *et al.*, 1995). This may help explain how the virus is able to escape the immune system and persist so effectively in the host. EBNA-2 is essential for immortalisation of B cells *in vitro* (Cohen *et al.*, 1989) and has been shown to transactivate the LMP genes and the cellular oncogenes c-*fgr* and *bcl*-2. EBNA-3A, B and C originate from a single open reading frame and their functions are less well characterised, although EBNA 3A and C are also necessary for immortalisation (Tomkinson *et al.*, 1993). LP is not essential for immortalisation but may have a potential role in controlling cell proliferation. LMP-1 is an integral membrane protein and a classical viral oncoprotein (Kaye *et al.*, 1993). As discussed in section 3.4.1, LMP-1 induces activation of the cellular transcription factor, NFκB, and up-regulates the anti-apoptotic protein, Bcl-2 (Henderson *et al.*, 1993). LMP-2A and 2B are also transmembrane proteins. LMP-2A interacts with the *src* family of tyrosine kinases and may be involved in suppressing reactivation (Miller *et al.*, 1995a). The EBERs are dispensable for viral replication but may have a role in transformation of Akata cells (Komano et al., 1999).

Latency type I is the pattern of expression detected in biopsy samples in cases of endemic BL. In these cells, only EBNA-1, the EBERs and the BARTS are expressed. Latency type II is observed in NPC and HD lymphomas (Brooks *et al.*, 1992; Deacon *et al.*, 1993; Hamilton-Dutoit *et al.*, 1993; Young *et al.*, 1988).

Expression is limited to EBNA-1, LMP-2A, LMP-2B, the BARTs and in some cases, LMP-1. In the persistently infected resting B cells of seropositive immunocompetent hosts, only LMP-2A and the EBERs are detectable.

1.3. Kaposi's Sarcoma-Associated Herpesvirus

1.3.1. The KSHV Genome

Kaposi's sarcoma herpesvirus (KSHV, HHV-8) was first identified using a technique known as representational difference analysis (Chang *et al.*, 1994; Lisitsyn & Wigler, 1993). This involves subtractive hybridisation of two different complex genomes. In this case, differences between DNA from a KS skin lesion and control tissue from the same patient led to the isolation of fragments of the KSHV genome. KSHV has since been fully cloned and sequenced and classified as a gammaherpesvirus of the genus, *Rhadinovirus* (Moore *et al.*, 1996b; Russo *et al.*, 1996). The unique region of the genome is 140.5kb in length and is interspersed with five internal repeat regions and flanked by multiple GC rich (85%) 801bp repeats. There are at least 81 ORFs, of which 66 share homology with herpesvirus saimiri. These include the full contingent of conserved herpesvirus genes, gammaherpesvirus-specific genes and a number of unique genes.

1.3.2. Diseases Associated with KSHV

Kaposi's sarcoma was previously a rare tumour affecting mainly middle-aged or elderly men in Mediterranean countries. The onset of the AIDS epidemic saw a vast increase in the number of cases and KS is now the most common neoplasm of AIDS patients, whose risk of developing the disease is 20,000 fold above the general population (Beral, 1991). There are four known epidemiological forms of KS: classic KS, AIDS-KS, endemic HIV-negative KS (Central Africa) and post-transplant associated KS. Classic KS is usually an indolent condition affecting the skin of the lower limbs, while endemic KS is associated primarily with the lymph nodes. AIDS-KS is a much more aggressive multi-system disease with a higher mortality rate.

KSHV sequences have been detected in all forms of KS, thus making it almost certainly the aetiological agent of Kaposi's sarcoma (Dupin *et al.*, 1995; Huang *et al.*, 1995; Moore & Chang, 1995; Schalling *et al.*, 1995). In addition, treatment of KS patients with the anti-herpetic drug, foscarnet, results in tumour regression, indicating that the disease is virus-driven (Morfeldt & Torssander, 1994). Unlike EBV, the seroprevalence of KSHV in the general population is low (0-1% of HIV-negative US blood donors). However, this figure rises to 35% in HIV^+/ KS^- homosexual men, suggesting that the disease is sexually transmitted (Blackbourn *et al.*, 1999; Kedes *et al.*, 1996). Seroconversion to KSHV is usually a prognostic indicator of KS development. Epidemiological studies have revealed high levels of KSHV seropositivity in Italian and Ugandan populations where the incidence of childhood KS provides evidence for vertical transmission (Gao *et al.*, 1996). Other researchers have also demonstrated the presence of KSHV in saliva, which may provide another route of transmission (Blackbourn *et al.*, 1998; Boldogh *et al.*, 1996; Koelle *et al.*, 1997; Vieira *et al.*, 1997).

KSHV is associated with a number of other malignancies including body cavity-based lymphoma (BCBL), also known as primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). As the name suggests, BCBL is a malignant lymphoma that is localised to the pleural or abdominal cavity and is generally associated with AIDS. KSHV DNA is observed in the majority of BCBL lymphomas (Cesarman *et al.*, 1995a), although the EBV genome is usually also present (Gessain, 1997). However, in some cases KSHV has been detected in the absence of EBV (Carbone *et al.*, 1996; Cesarman *et al.*, 1996b). The presence of the KSHV genome has also been demonstrated in MCD, another AIDS-related lymphoma (Soulier *et al.*, 1995).

1.3.3. KSHV Tropism and Gene Expression

Like EBV, KSHV is a B-cell tropic virus and several latently infected B cell lines have been derived from the peripheral blood mononuclear cells of BCBL patients (Cesarman *et al.*, 1995b; Cesarman *et al.*, 1996; Gaidano *et al.*, 1996; Renne *et al.*, 1996). However, KS is a vascular tumour consisting of bundles of spindle cells,

which belong to the endothelial lineage but often express markers characteristic of endothelial, macrophage and smooth muscle cells. *In situ* hybridisation analysis has detected the presence of KSHV-specific transcripts in the majority of these cells (Staskus *et al.*, 1997). There is a restricted pattern of KSHV gene expression in both spindle cells and in B-cell lymphoma derived cell lines such as BC-1, indicative of a latent infection. As with EBV^+ cell lines, lytic replication can be induced by addition of phorbol esters or sodium butyrate (Miller *et al.*, 1997; Miller *et al.*, 1996). Transcriptional analysis of KSHV in the BC-1 cell line has lead to the grouping of KSHV genes into three classes: constitutively transcribed latent transcripts that are unaffected by phorbol ester (class I); genes that are up-regulated by phorbol ester and sodium butyrate (class II); transcripts that are only detectable following induction (class III, Sarid *et al.*, 1998). Class I genes include the latency associated nuclear antigen (LANA), a DNA-binding protein which is thought to have a function similar to that of EBV EBNA-1. In addition, KSHV encodes a relatively large number of cellular homologues (discussed in later section), two of which, v-cyclin and v-FLIP, are transcribed during latency. The KSHV GPCR (ORF74) is a class III gene, with transcripts only observable following induction of productive infection in latent cell lines (Talbot *et al.*, 1999).

1.4. Other Gammaherpesviruses

1.4.1. Herpesvirus Saimiri

Herpesvirus saimiri (HVS) is a T-cell tropic rhadinovirus whose natural host is the squirrel monkey. In other species of New World primate, HVS infection leads to the development of fatal T cell lymphomas (Desrosiers, 1981; Desrosiers *et al.*, 1985; Fleckenstein, 1979; Wright *et al.*, 1977). The unique region (112.9kb) of the HVS genome is flanked by 1.4kb GC rich repeats and contains at least 76 ORFs, of which 60 are encoded by other herpesviruses. Three strains of HVS (A, B, C) have been distinguished on the basis of sequence variation in the leftmost region of the unique sequence, which encodes the HVS transforming genes (Medveczky *et al.*, 1984). Strains A and C are highly oncogenic and both transform common marmoset T lymphocytes to IL-2-independent growth *in vitro* (Desrosiers *et al.*, 1985; Duboise *et*

al., 1998a; Jung *et al.*, 1991). In addition, strain C has the capacity to immortalise human, rabbit and rhesus monkey cells into continuously proliferating T cell lines (Ablashi *et al.*, 1985; Mittrucker *et al.*, 1993). Strain C is also more oncogenic than A and B *in vivo*: it produces lymphomas in rabbits whereas the other strains do not (Medveczky *et al.*, 1989). The existence of a permissive tissue culture system has made HVS a popular model for investigating virus-mediated oncogenicity.

1.4.2. Alcelaphine Herpesvirus 1 and Ovine Herpesvirus 2

Alcelaphine herpesvirus 1 (AHV-1) is the causative agent of malignant catarrhal fever virus (MCF), a lymphoproliferative disease of ruminants. AHV-1 is asymptomatic in its natural wildebeest host but gives rise to MCF in cattle and deer, creating a significant economic impact. Cloning and sequencing of the AHV-1 genome revealed it to be most closely related to HVS (Ensser *et al.*, 1997). Epidemiological evidence implicated sheep as asymptomatic carriers of MCF and analysis of lymphoblastoid cell lines derived from infected cattle detected the presence of AHV-1-like DNA sequences, now known to belong to ovine herpesvirus 2 (Bridgen & Reid, 1991)

1.5. Murine Gammaherpesvirus 68

1.5.1 MHV-68 as an Animal Model

Murine gammaherpesvirus 68 is a naturally occurring gammaherpesvirus, closely related to the clinically important gammaherpesviruses, EBV and KSHV. The restricted host range of these viruses and their limited ability to grow *in vitro* has hampered studies of EBV and KSHV. Studies of gammaherpesviruses in humans are constrained by the frequently asymptomatic nature of the acute infection and the delay between infection and presentation in patients with disease. Likewise, animal models of EBV-mediated lymphoproliferative disease such as rhesus macaques, common marmosets and cottontop tamarins do not mirror the human condition (Finerty *et al.*, 1992; Moghaddam *et al.*, 1997). Primary infection of marmosets (*Callithrix jacchus*) with EBV gives rise to symptoms similar to infectious mononucleosis, however there is no B cell proliferation and no detectable EBV-

specific proteins in the peripheral blood lymphocytes (Emini *et al.*, 1986). In cottontop tamarins, high doses of EBV engendered lethal lymphomas within 14-21 days, thus providing little information about chronic infection. Infection of chimpanzees via contact with humans has been reported but their limited availability precludes their use as an effective model (Levine *et al.*, 1980). Two small animal models involving EBV-related herpesviruses have also been proposed. Rabbits infected with the baboon (*Macaca arctoides*) herpesvirus (HVMA) or the cynomolgus (cyno-EBV), exhibit malignant lymphomas (Hayashi & Akagi, 2000; Wutzler *et al.*, 1995). However, the tumours produced are primarily T-cell lymphomas whereas EBV generates predominantly B-cell lymphomas. Lastly, a mouse model has been proposed that involves reconstitution of severely combined immunodeficient mice (SCID) with human EBV^+ lymphocytes (Johannessen & Crawford, 1999; Mosier *et al.*, 1992; Rowe *et al.*, 1991). SCID/hu-PBL-SCID mice rapidly develop multiple monoclonal or oligoclonal lymphomas, which bear similarity to the large-cell lymphomas observed in patients with post-transplant lymphoproliferative disease. Criticisms of this model have included the unnatural route of infection and the cross species differences that may influence the pathology of the virus. There is also a lack of an animal model for KSHV. As with EBV, the SCID mouse has been proposed as a model: SCID mice were implanted with human foetal or liver tissue inoculated with KSHV virions; but although the mice displayed evidence of viral gene transcription, principally in B cells, there were no disease symptoms (Dittmer *et al.*, 1999).

The pathogenesis of MHV-68 in laboratory mice resembles that of EBV in humans. It produces an acute infection similar to infectious mononucleosis and is also associated with lymphoproliferative disease. Unlike EBV and KSHV, MHV-68 initiates a productive infection in a wide range of tissue culture cells and can be grown to high titres. It is also possible, using molecular techniques, to manipulate the virus genome *in vitro* and investigate the effects *in vivo*. Therefore MHV-68 provides an amenable small animal model for the study of gammaherpesvirus infection and associated lymphomagenesis in a natural host system.

1.5.2. The MHV-68 Genome

MHV-68 is one of five viruses that were originally isolated from the bank vole, (*Clethrionomys glareolus*) and the yellow-necked mouse (*Apodemus flavicollus*) in Slovakia (Blaskovic *et al.*, 1980). Initially considered to possess the growth characteristics of an alphaherpesvirus (Svobodova *et al.*, 1982), MHV-68 was later classified as a gammaherpesvirus on the basis of structural and sequence homology. Limited sequence analysis revealed a number of open reading frames with greatest similarity to the homologous proteins of HVS and EBV (Efstathiou *et al.*, 1990a; Mackett *et al.*, 1997). It also showed that the MHV-68 genome exhibited CpG dinucleotide suppression, a common feature of gammaherpesviruses thought to arise from the mutagenic effect of methylation during virus latency (Bird, 1980; Honess *et al.*, 1989). In addition, the organisation of the MHV-68 genome was found to be collinear with that of HVS and EBV genomes (Gompels *et al.*, 1988). Cloning of the double-stranded DNA genome showed that it consists of 118.2kb of unique DNA sequence flanked by a variable number of copies of a 1.23kb terminal repeat. The DNA sequence of the genome has now been fully determined, revealing the presence of two internal repeats of 40bp and 100bp (Virgin *et al.*, 1997; Davison, in press). The GC content of the MHV-68 unique region (46%), like HVS (35%) and KSHV (54%), is lower than that of EBV (60%), whereas the MHV-68 terminal repeats are very GC rich (78%) (Albrecht *et al.*, 1992a; Russo *et al.*, 1996). The MHV-68 unique region consists of large blocks of conserved genes, interspersed with virus-specific genes. Approximately 80 open reading frames (ORFs) have been determined, of which 63 are homologues of HVS or KSHV genes. On the whole, the identity of MHV-68 ORFs to those of other gammaherpesviruses indicates that MHV-68 is more closely related to HVS and KSHV than to EBV, indicating that MHV-68 belongs to the gamma-2 subfamily.

A schematic representation of the MHV-68 genome is given in figure 1.5.2. The conserved herpesvirus family genes include the capsid proteins and the glycoproteins, gB, gH, gM and gL; genes involved in nucleic acid metabolism such as uracil DNA glycosylase, deoxyuracil triphosphatase and large subunit of ribonucleotide reductase; DNA replication enzymes e.g. DNA polymerase and

Figure 1.5.2. The MHV-68 genome. A single dark blue line represents the unique region with the genome co-ordinates marked at 10kb intervals. The position of the terminal repeats (TR) and internal repeats (IR) is indicated by dark blue boxes. The open reading frames (ORFs) are drawn as arrows pointing in the direction of transcription. Yellow arrows signify genes that are unique to the MHV-68 genome, while blue arrows denote the MHV-68 glycoproteins. The genes are labelled according to function: *crp*, complement regulatory protein; *gB*, glycoprotein B; K3, homologue of the KSHV immediate early gene; *TK*, thymidine kinase; *gH*, glycoprotein H; *gM*, glycoprotein M; *ung*, uracil DNA glycosylase; *gL*, glycoprotein L; *rta*, R (immediate early) transcriptional transactivator; *gp150*, glycoprotein with homology to EBV gp340/220; *dut*, dUTPase; *mta*, M (immediate early) transcriptional transactivator; *rr2*, small subunit ribonucleotide reductase; *rr1*, large subunit ribonucleotide reductase; *cyc D*, homologue of mammalian cyclin D; *bcl-2*, homologue of mammalian bcl-2; GPCR, homologue of mammalian CXCR2. Where the function of the gene is unknown, it is represented by the ORF number. The MHV-68 GPCR is situated at the right-hand end of the genome and is labelled orange. Adapted from Stewart, 1999a.

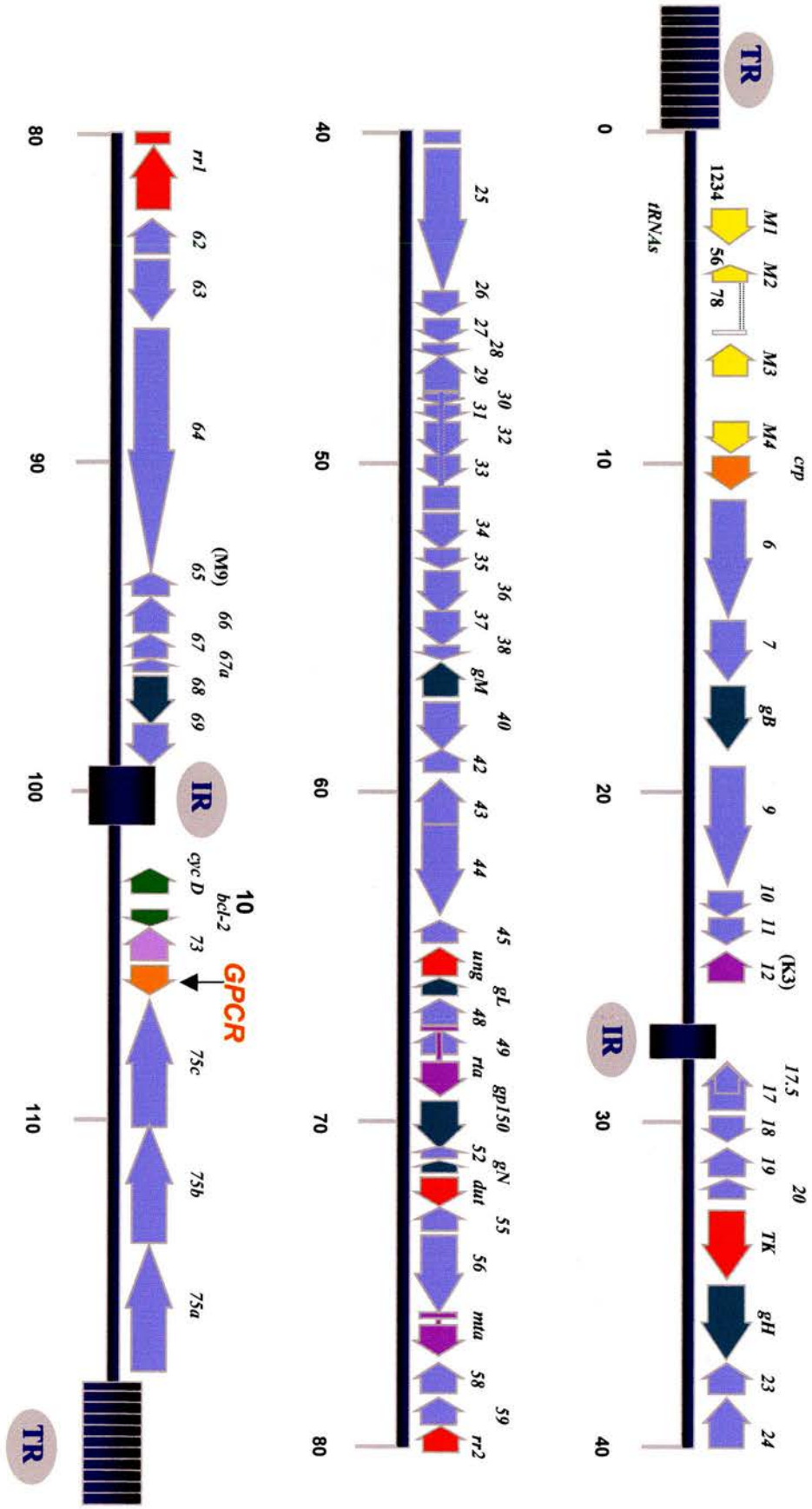


Figure 1.5.2. The MHV-68 Genome

helicase-primase. The semi-conserved genes include thymidine kinase and the small subunit of ribonucleotide reductase. Unique genes are prefaced by the initial, M.

1.5.3. MHV-68 Gene Expression

During the 24-hour productive cycle, MHV-68 genes are expressed in a conventional cascade beginning with immediate early genes such as ORF50 (*rta*), early genes (e.g. thymidine kinase) and late genes (e.g. glycoprotein 150). Gene expression during latent infection is less clear but MHV-68 does not appear to encode homologues of the HVS or EBV latency-associated genes (Virgin *et al.*, 1997). However, MHV-68 does express eight tRNA molecules during latency, which are reminiscent of the EBV EBER sequences. The M2 gene is expressed during latent infection both *in vivo* and in the MHV-68 positive lymphoma cell line, S11, which has proved to be a valuable model for investigating virus latency (Husain *et al.*, 1999). The M3 gene, however, is not expressed in S11 cells but can be detected in the spleens of chronically infected mice (Simas *et al.*, 1999). Therefore, like EBV, MHV-68 may have several forms of latency. Additionally, multiple spliced transcripts crossing the terminal repeats have been detected in S11 cells (Husain *et al.*, 1999). Expression of known lytic genes such as bcl-2, ORF73 and the GPCR has also been observed in the absence of other lytic transcripts thus indicating a role in latent infection and virus persistence (Virgin *et al.*, 1999).

1.5.4. Pathogenesis of MHV-68 Infection

The natural route of MHV-68 infection is unconfirmed but most likely involves close contact and respiratory transmission. Intranasal inoculation results in productive infection of alveolar epithelial cells and mononuclear cells in the lung causing in a severe exudative pneumonia and haematogenous dissemination of the virus to other tissues including kidneys, adrenal glands, heart and spleen depending on the age of the mice and the route of infection (Blaskovic *et al.*, 1984; Rajcani *et al.*, 1985). Inflammation and lymphocyte infiltration are observed in the lung during the course of the acute infection, which peaks around 5 days post-infection and is cleared by day 14. At this point, the virus is undetectable by conventional assays in every tissue except the spleen, which is a major site of viral persistence (Sunil-Chandra *et al.*,

1992a; Usherwood *et al.*, 1996b). Latency is established in splenic B-lymphocytes, as confirmed by the absence of splenic latency in μMT B cell-deficient transgenic mice (Usherwood *et al.*, 1996a). Latently infected B cells begin to appear during the first week of infection and peak around the second or third week in a dramatic splenomegaly similar to that observed during EBV-induced infectious mononucleosis.

There is evidence that the lung is also a major site of viral persistence: viable episomal MHV-68 DNA is detectable in the lung long after acute infection by PCR in both normal and B cell-deficient μMT mice. Latency-associated transcripts, such as M2, are also detectable months after the acute infection. In addition, adoptive transfer of B cells into infected μMT mice facilitates infection of splenic B cells by persistent virus in the lung, suggesting that the lung is functioning as a non-lymphoid virus reservoir (Stewart *et al.*, 1998). *In situ* hybridisation studies demonstrated that latent virus persists in pulmonary epithelial cells. Macrophages harvested from the peritoneal exudate of μMT mice infected intra-peritoneally (IP) with MHV-68, have also been reported to harbour latent virus (Weck *et al.*, 1999). Therefore, MHV-68 is unlike EBV in that it establishes latency in epithelial cells in addition to B cells. That said, EBV has been associated with a chronic form of lung disease, cryptic fibrosing alveolitis, suggesting that the lung may act as a reservoir for EBV (Egan, 1995). Since MHV-68 also appears to be the causative agent of arteritis in chronically infected mice and splenic fibrosis in interferon gamma receptor knockout mice, it may prove a useful model in investigating this aspect of gammaherpesvirus pathogenesis (Weck *et al.*, 1997; Dutia *et al*, 1997).

1.5.5. Immune Response to MHV-68 Infection

Acute infection induces a large inflammatory infiltrate in the lung. At 3-4 days post-infection, this is comprised primarily of mononuclear phagocytes, as shown by analysis of the bronchoalveolar lavage of infected mice (Sunil-Chandra *et al.*, 1992a). However, the monocytes are replaced by large numbers of $CD8^+$ cytotoxic T cells, which peak around day 8-10. There is a predominance of $CD8^+$ T cells with a Vβ4 phenotype, which suggests that a viral superantigen may stimulate selective

expansion of this subset (Doherty *et al.*, 1997). Depletion of T-cell subsets has shown that $CD8^+$ T cells appear to be crucial to the resolution of the acute infection, whereas the absence of $CD4^+$ T cells provokes only a slight delay in virus clearance (Nash *et al.*, 1996). However, while $CD4^+$ T cells do not dramatically influence the course of infection of the lung, they contribute to lymphoproliferation in the spleen. Depletion of $CD4^+$ T cells prevents the development of splenomegaly and greatly reduces the level of latent virus that is normally observed during the peak of splenomegaly at 2-3 weeks post-infection (Usherwood *et al.*, 1996a). Splenomegaly also fails to occur in $CD4^+$ T-cell and B-cell deficient mice (Ehtisham *et al.*, 1993; Usherwood *et al.*, 1996b). While $CD4^+$ T cells are essential for the development of splenomegaly, $CD8^+$ T cells influence the subsequent fall in cell number, as demonstrated by the inability of β_2-microglobulin-deficient mice to clear infectious virus from the spleen (Weck *et al.*, 1996). This suggests that although $CD8^+$ T cells reduce the number of infected B cells in the spleen, some infected B cells escape recognition and establish a lifelong latent infection.

There is only a slight delay in resolution of the acute infection in the lung in B-cell deficient mice, indicating that antiviral antibody does not play an important role in virus clearance. Even so, neutralising antibody levels remain high throughout the lifetime of the animal (Stevenson & Doherty, 1998).

Mice infected intranasally with MHV-68 produce high levels of interferon gamma (IFNγ) and interleukin 6 (IL-6) in lymphoid tissues, with levels peaking around day 10 post-infection. Low levels of interleukin 10 (IL-10) and interleukin 2 (IL-2) are also produced (Sarawar *et al.*, 1996). IFNγ and IL-2 stimulate $CD8^+$ T cells that are critical for virus clearance. Paradoxically, IFNγ and IL-6 stimulate B-cell proliferation, which may serve to increase the number of latently infected cells. IFNγ does not appear to be essential for viral clearance as transgenic IFNγ deficient mice resolve the acute infection almost as efficiently as wild type mice (Sarawar *et al.*, 1997). However, mice lacking the IFNγ receptor exhibit splenic atrophy when infected with MHV-68 and display a vast decrease in the number of splenic B and T cells (Dutia *et al.*, 1997). Type I interferons (interferon alpha and interferon beta)

have been shown to be vital for clearance of the virus as IFNα/β receptor deficient mice succumb to a fatal MHV-68 infection. Thus, both the adaptive and innate arms of the immune response are critical in controlling MHV-68 infection.

1.5.6. Lymphoproliferative Disease Associated with MHV-68

Long-term infection of inbred mice with MHV-68 is associated with development of tumours (Sunil-Chandra *et al.*, 1994). Lymphoproliferative disease occurred in 9% of chronically infected mice over a period of 9 months to 3 years following intranasal infection with MHV-68, of which 50% were high-grade lymphomas. Lymphomas were associated with both lymphoid and non-lymphoid tissue (lung, liver and kidney). The number of mice developing lymphoproliferative disease rose to 60% when treated with the immunosuppressive drug, cyclosporin A. The lymphomas were of mixed B and T cell origin: in all cases the B cells were light chain restricted, suggesting a clonal origin; T cells were $CD3^+$ and belonged predominantly to the $CD4^+$ subset. MHV-68 DNA was detected in the absence of lytic antigen in a variable number of cells in and around the lymphomas, thus indicating that the cells were latently infected.

The virus infects myeloma cells (NS0, B cell lineage) *in vitro* and a state of latency is achieved in which the virus genome exists in an episomal form (Sunil-Chandra *et al.*, 1993). Alternatively the B cell line, S11, was generated from spleen cells of a chronically infected mouse showing evidence of lymphoma development. S11 cells contain the MHV-68 genome in both episomal and linear form, although suppressing lytic replication via acyclovir treatment can increase the ratio of circular to linear genomes (Usherwood *et al.*, 1996c).

1.6. Viruses and Immune Evasion

It is not uncommon for viral genomes to encode homologues of mammalian genes, presumably acquired via a recombination event and conserved because they confer a selective advantage. There are many such examples of "molecular piracy" among members of the herpesvirus and the poxvirus family. Many of the cellular homologues present in these viruses tend to be genes involved in immune regulation

and such genes appear to confer an obvious advantage to the virus in escaping immune surveillance and establishing a persistent infection.

1.6.1. Chemokine Binding Proteins

Chemokine binding proteins are a feature of several virus genomes and are particularly prevalent amongst poxviruses. These are generally abundantly secreted proteins that compete with cellular chemokine receptors for chemokines, thus "mopping-up" the inflammatory response. For instance, vaccinia and cowpox virus encode soluble homologues of the IL-1β receptor that appear to enhance host survival, and hence viral survival, by protecting against the adverse effects of excessive cytokine production (Alcami & Smith, 1992; McMahan *et al.*, 1991; Smith & Chan, 1991; Spriggs *et al.*, 1992). Myxoma virus secretes a glycoprotein (CBP-1) that interacts with the conserved heparin-binding domain of CXC, CC and C chemokines. Additionally, several poxviruses (variola, cowpox, vaccinia and Shope fibroma virus) encode a soluble chemokine inhibitor (vCCI) with no significant homology to cellular proteins that binds virtually all known CC chemokines and reduces the influx of inflammatory cells into virus-infected tissues (Graham *et al.*, 1997; Smith *et al.*, 1997). Soluble receptors for type I and type II interferons have also been reported, which have the ability to modulate the IFN-induced antiviral state (Alcami & Smith, 1996). There is also a chemokine-like molecule encoded by the human poxvirus, *Molluscum contagiosum*, which has no intrinsic chemotactic ability but antagonises cellular chemokines and has an inhibitory effect on human haematopoietic progenitor cells (Krathwohl *et al.*, 1997). Examples of broad-spectrum chemokine-like antagonists have also been discovered in HHV-6, MCMV, HCMV and KSHV (Nicholas *et al.*, 1997; Penfold *et al.*, 1999; Saederup *et al.*, 1999; Zou *et al.*, 1999). The MHV-68 soluble protein, M3, has recently been designated a chemokine-binding protein. M3, which is detectable during latent infection *in vivo*, blocked the action of a more specific range of chemokines: RANTES, MIP-1α, MCP-1, IL-8, lymphotactin and fractalkine (Parry, et al., 2000; van Berkel *et al.*, 2000).

1.6.2. Cytokine Homologues

HVS encodes several genes involved in immunomodulation: the ORF13 product has 72% identity with human IL-17, which is produced by $CD4^+$ T cells. Like hIL-17, vIL-17 activates the transcription factor, NFκB, stimulates IL-6 secretion in fibroblasts, and co-stimulates T cell proliferation, thereby providing a selective advantage to a T-lymphotropic virus (Yao *et al.*, 1995).

The EBV genome includes a homologue of IL-10, a pleiotropic cytokine that inhibits the production of IL-2 and IFNγ and promotes the growth of B cells. The virus homologue is 70% identical to its human counterpart and also functionally analogous. vIL-10 enhances EBV-driven transformation of B cells. This effect is likely to be mediated by increasing the number of viable B cells and reducing the levels of IFNγ, a cytokine known to inhibit EBV transformation (Stuart *et al.*, 1995).

KSHV encodes four cytokine homologues: three with homology to mammalian macrophage inflammatory protein 1α (MIP-1α), and one that is most closely related to IL-6 (Moore *et al.*, 1996a; Neipel *et al.*, 1997). vMIPII binds a number of cellular chemokine receptors but fails to activate any signalling pathways, suggesting that it acts as a competitive antagonist (Kledal *et al.*, 1997). Consistent with this, vMIPII is a potent inhibitor of CC and CX_3C chemokine-induced chemotaxis *in vitro* (Chen *et al.*, 1998). Both vMIPI and vMIPII induce angiogenesis *in vitro* while vIL-6 has a proliferative effect on myeloma cells, thus strengthening the theory that deregulated cytokine production is an important feature in the development of KS (Boshoff *et al.*, 1997; Burger *et al.*, 1998). Furthermore, the discovery of an interferon regulatory factor (vIRF) that suppresses type I IFN-induced gene expression and has a transforming activity *in vitro*, indicates that KSHV escapes the immune response by antagonising the IFN-mediated antiviral pathway (Li *et al.*, 1998).

1.6.3. Regulators of Complement Activation

HVS possesses a homologue of mammalian CD59, a molecule involved in T cell activation and restriction of complement-mediated lysis, that may offer protection to infected cells (Albrecht *et al.*, 1992b). The complement control protein homologue

(CCPH) reduces complement-mediated lysis by inhibiting C3 convertase and down-regulating deposition of the activated complement component, C3d, on the cell surface (Fodor *et al.*, 1995). CCPH is an alternatively spliced gene producing soluble and membrane-bound isoforms. Positional homologues of CCPH are present in MHV-68 and KSHV, both encoded by ORF4 (Russo *et al.*, 1996; Virgin *et al.*, 1997). The MHV-68 CCPH, like the HVS CCPH, inhibits cell-surface deposition of C3d (Kapadia *et al.*, 1999).

1.6.4. Super-Antigens

HVS ORF14 shares 22% amino acid identity with the mouse mammary tumour virus (MMTV7) super-antigen. The ORF14 product is a secreted protein that binds MHC class II and stimulates T cell proliferation (Yao *et al.*, 1996). The selective expansion of $V\beta4^+/CD8^+$ T cell subset during MHV-68 infection is indicative of the action of an as yet uncharacterised viral super-antigen (Tripp *et al.*, 1997).

1.6.5. Mammalian IL-8 and its Receptors

Several herpesviruses encode homologues of the mammalian chemokine receptor, CXCR2, which belongs to the superfamily of G protein-coupled receptors (GPCRs). Many of these viral homologues have been found to be functional chemokine-binding signalling molecules. Viral GPCRs are discussed in detail in the subsequent sections but there firstly follows a description of their closest cellular homologue, CXCR2.

The chemokines are a superfamily of small, structurally related cytokines that have been shown to promote the rapid adhesion, chemotaxis and activation of leukocyte effector subpopulations. Chemokines are classified as C, CC, CXC or CX_3C, based on the position of well-conserved cysteine residues. The CC chemokines include RANTES, monocyte chemotactic protein (MCP-1) and MIP-1. The CXC chemokines include IL-8, platelet factor 4 (PF4), neutrophil-activating peptide (NAP-2) and growth-related oncogene (GROα). Lymphotactin and fractalkine are the sole respective members of the C and CX_3C groups.

As the name suggests, CXCR2 binds CXC chemokines such as IL-8. IL-8 is a pro-inflammatory chemokine, which is secreted from many cell types in response to stimulation by IL-1β, TNFα and lipopolysaccharide. It has also been reported that EBV-immortalised lymphoblastoid cell lines, some neoplastic B cells and KS spindle cells produce appreciable levels of IL-8 (di Celle, 1996; Merico *et al.*, 1993; Sciacca *et al.*, 1994).

Binding of IL-8 to neutrophils results in a transient intracellular calcium flux which signals changes in morphology, increased motility and transvenule migration (Huber *et al.*, 1991). Neutrophil effector mechanisms are rapid and antigen independent. Degranulation results in the release of elastase and myeloperoxidase and a respiratory burst gives rise to hydrogen peroxide and superoxide radicals, all of which contribute to destroying pathogens. IL-8 is also a regulator of angiogenesis (Maione *et al.*, 1990). Angiostatic activity has been reported for other CXC chemokines such as PF-4 and GROα but IL-8 possesses an ELR motif that confers angiogenic activity (Strieter *et al.*, 1995).

Two human IL-8 receptors have been identified, CXCR1 and CXCR2 that are 77% identical at the amino acid level. CXCR1 binds only IL-8 with high affinity whereas CXCR2 is a more promiscuous receptor with affinity for GROα and NAP-2 (Holmes *et al.*, 1991; Murphy & Tiffany, 1991). Expression of CXCR2 is confined to neutrophils, neutrophil cell lines and myeloid-precursor cell lines. CXCR1 is expressed on a much wider range of cells: PHA-activated T cell blasts, $CD4^+$ cells, monocytes and monocyte-like cell lines, melanoma cells, synovial fibroblasts in addition to the myeloid cells which express CXCR2. Both human genes map closely together on chromosome 2q35 along with a pseudogene for CXCR2. All three genes probably arose as the result of a duplication event.

Mice encode only one gene with a high degree of homology to the two human receptors for IL-8 (Lee *et al.*, 1995). Although no rodent IL-8 has yet been identified, human IL-8 induces chemotaxis of murine neutrophils and antibodies to human IL-8 inhibit lung inflammation in the rat. This suggests that the absence of

IL-8 is compensated by similar chemokines such as mouse MIP-2 (Tekamp-Olson *et al.*, 1990) and KC (Oquendo *et al.*, 1989). Transgenic mice in which the receptor has been deleted are outwardly healthy but exhibit lymphadenopathy and splenomegaly indicative of increased B cells, melanocytes and neutrophils. The murine chemokine receptor may therefore have a negative regulatory role in B cell and neutrophil development. The migrational ability of neutrophils to sites of inflammation was also severely compromised (Cacalano *et al.*, 1994).

1.7. G Protein-Coupled Receptors

The guanine nucleotide binding (G) protein-coupled receptors (GPCRs) superfamily of receptors contains almost 200 members, which are involved in a wide range of processes such as neurotransmission, vision, olfaction and cardiac output. GPCRs respond to such functionally diverse ligands as small biogenic amines (e.g. adrenaline), chemokines (e.g. IL-8), neuropeptides (e.g. bradykinin) and large glycoprotein hormones (e.g. luteinising hormone and parathyroid hormone).

1.7.1. GPCR Structure

Despite their functional diversity, GPCRs share a common structure characterised by seven transmembrane domains, an extracellular N-terminus and a cytoplasmic C-terminus (Gether & Kobilka, 1998). The conserved transmembrane domains contain 20-25 hydrophobic residues with an α-helical structure and are connected by divergent hydrophilic loops. Evidence from X-ray diffraction studies of bacterial rhodopsin indicates that the transmembrane domains are arranged in a barrel-like structure with a tightly packed core (Pebay-Peyroula *et al.*, 1997; Peitsch *et al.*, 1996). Mutational analysis of two chemokine receptors (CXCR4 and CCR5) has revealed that five transmembrane domains (TM3-7) are sufficient for cellular expression, signalling, internalisation and desensitisation (Ling *et al.*, 1999). However, this specificity is likely to be limited to certain GPCR subtypes since other receptors such as the adenosine A1 receptor require TM1 and TM2 for ligand binding (Casey & Gilman, 1988).

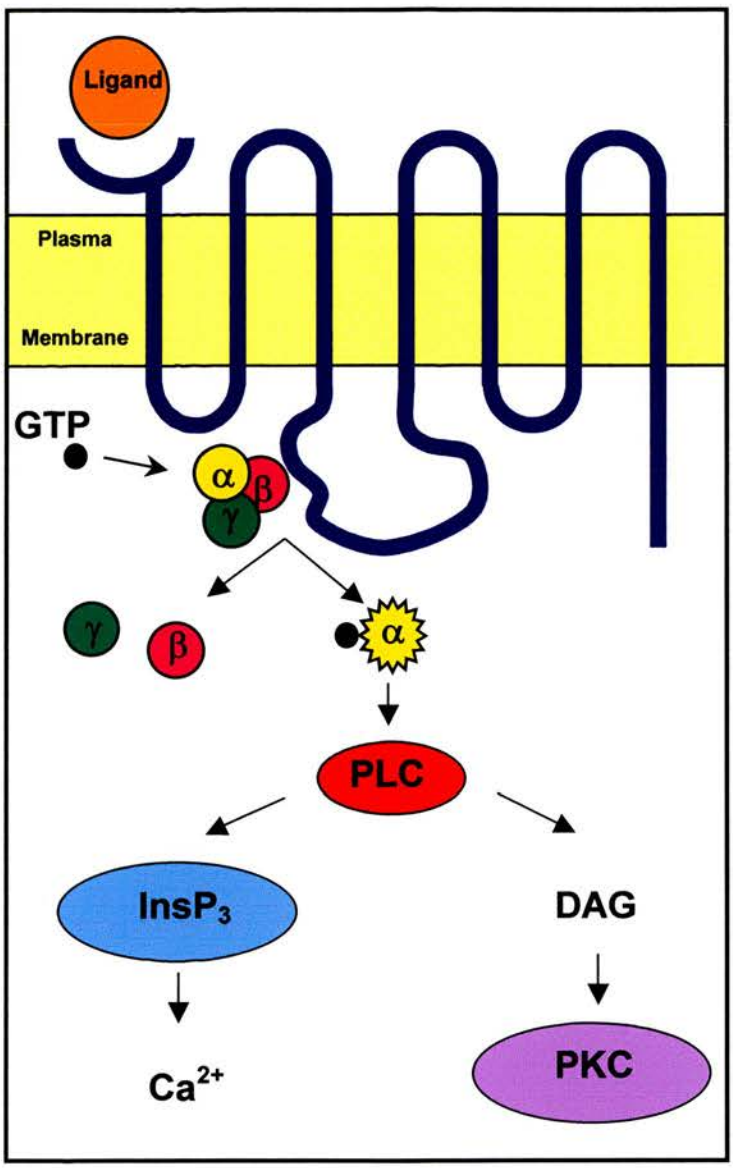
The majority of GPCRs are N-glycosylated at a consensus site (Asn-X-Ser/Thr) in the N-terminal region, a modification that appears to affect the level of receptor expression but not binding of the ligand (Strader *et al.*, 1995). GPCRs invariantly possess conserved cysteine residues that form a disulphide bridge linking the second and third intracytoplasmic loops. This is thought to be critical for maintaining the active conformation of the receptor (Karnik *et al.*, 1988). Conserved serine and threonine residues in the C-terminus are thought to be involved in receptor desensitisation.

Deletion analysis of the α_{1b}-adrenergic receptor, β_2-adrenergic receptor and the angiotensin II receptor has revealed the importance of residues in the 2nd and 3rd intracellular loops of GPCRs for coupling of G proteins (Samama *et al.*, 1993; Scheer *et al.*, 1996; Scheer *et al.*, 1997). In particular, a well-conserved motif, DRY, occurs at the beginning of the second intracytoplasmic loop. Mutations in this consensus motif are associated with constitutive signalling and oncogenicity. For instance, the agonist-independent KSHV GPCR encodes a VRY motif at this position and mutation of the CXCR2 DRY sequence to VRY results in constitutive signalling and confers oncogenic properties. It is thought that substitution of the aspartate residue with the hydrophobic valine alters the receptor structure by drawing the loop into the plasma membrane, thus constraining the GPCR in an active conformation (Burger *et al.*, 1999). Further examination of the DRY motif in the histamine (H2) receptor reinforced the importance of the aspartate and arginine residues. Substitution of the aspartate residue resulted in high-level constitutive signalling and increased agonist-affinity. Interestingly, mutations in these amino acids also resulted in structural instability (Alewijnse *et al.*, 2000).

1.7.2. GPCR Signalling

A common property of GPCRs is that upon activation they transmit signals across the plasma membrane via an interaction with heterotrimeric G proteins (Stadel *et al.*, 1997). The signalling pathway shown in figure 1.7.2 is initiated by binding of the specific ligand which, depending on the GPCR, may bind to the TM core, the TM core and extracellular loops, extracellular loops and N-terminus, or exclusively to the

Figure 1.7.2. G Protein-Coupled Receptor Signalling



G protein-coupled receptors transduce signals via their interaction with G proteins. Ligand binding elicits a conformational change in the second intracellular loop of the receptor that facilitates coupling of the heterotrimeric G protein. This results in GTP hydrolysis, which leads to disassociation of the activated alpha (α) subunit from the beta/gamma (β/γ) complex. The alpha subunit binds to a second messenger such as phospholipase C (PLC) thus triggering a signalling cascade. PLC breaks down into inositol phosphate ($InsP_3$) and diaglycerol (DAG) which in turn mobilise intracellular calcium and protein kinase C (PKC).

N-terminus. This results in a conformational change in the receptor cytoplasmic domain, allowing interaction of heterotrimeric G proteins with binding sites in the second and third intra-cytoplasmic loops. The high-affinity agonist-receptor-G protein complex catalyses guanine nucleotide exchange on the α subunit leading to its dissociation from the β and γ subunits. The active GTP-bound α subunit then dissociates from the receptor and stimulates an intracellular effector molecule such as adenylate cyclase, guanyl cyclase, phospholipases A_2, C and D, calcium and potassium channels (Strader *et al.*, 1995). The GPCR may be desensitised by GPCR-specific kinases or second messenger-activated protein kinases (e.g. protein kinase C).

A major question regarding the sheer number and diversity of GPCRs is how the specificity of the signal is determined. Some specificity is achieved via ligand binding but since some GPCRs bind multiple ligands, a degree of specificity is also mediated by G protein-coupling. At least twenty different Gα, five Gβ and eleven Gγ subunits have been identified so far (Ray *et al.*, 1995; Simon *et al.*, 1991). The Gα subunits can be subdivided (G_s, G_i, G_q) according to the pathways they activate and GPCRs can be grouped according to the particular G protein subtypes they predominantly interact with. Thus, receptors that couple to G_i proteins stimulate adenylate cyclase while G_q proteins mobilise intracellular calcium via activation of phospholipase C. Nevertheless, there are some cellular GPCRs that can activate more than one Gα subtype (Eason & Liggett, 1995; Laugwitz *et al.*, 1996) and in general, GPCRs display promiscuity for different Gα subunits in reconstitution experiments. This potential for a large number of interactions between G proteins and a single GPCR can therefore result in multifunctional signalling (Milligan, 1993). There is some evidence to suggest that the G$\beta\gamma$ subunits, which show only limited coupling to other GPCRs in this type of experiment, are the more important determinants of specificity. Also, targeting of GPCRs to specific membranes allows the selectivity to be achieved by compartmentalisation of signalling components (Neubig, 1998). Another dimension of GPCR signalling is their ability to function synergistically. There are several examples, particularly among neurotransmitters, where GPCR cross-talk results in amplification of a signal. For instance, the

contractile effect of noradrenaline on blood vessels can be augmented by binding of neuropeptide Y or serotonin to their GPCRs. In addition, GPCRs may also interact with other receptor classes such as receptor tyrosine kinases to enhance signalling (Selbie & Hill, 1998).

1.7.3. Oncogenicity of GPCRs

Many GPCRs are involved in growth and differentiation and have been shown to be oncogenic in some contexts. For instance, the human *mas* oncogene was found to encode the angiotensin receptor, a member of the GPCR family (Young *et al.*, 1986). Transfection of the serotonin 5HT1c receptor into NIH3T3 cells results in the generation of transformed cellular foci, which produce tumours when injected into nude mice (Julius *et al.*, 1989). Similarly, a constitutively activating mutation in the α_{1B}-adrenergic receptor enhances mitogenesis and tumorigenicity in Rat-1 and NIH3T3 cells (Allen *et al.*, 1991). The muscarinic acetylcholine receptor subtypes have also been described as proto-oncogenes (Gutkind *et al.*, 1991). The initial transformation event appears to be agonist-dependent in the case of the serotonin receptor and muscarinic acetylcholine receptors whereas transformation by the α_{1B}-adrenergic receptor is merely agonist-enhanced.

1.7.4. GPCRs and Disease

The ubiquitous nature of GPCRs ensures their involvement in a wide range of diseases. For instance, naturally occurring mutations in cellular GPCRs such as the thyrotropin receptor and PTH-PTHrP receptor have been linked with thyroid adenomas and Jansen-type metaphyseal chondrodysplasia respectively (Parma *et al.*, 1993; Schipani *et al.*, 1995; Van Sande *et al.*, 1995).

Several GPCRs appear to have an involvement in HIV pathogenesis. Although CD4 is the primary cell surface receptor for HIV, a number of chemokine receptors have been identified that act as co-receptors for the virus, thus reflecting the extended tropism of non-syncytium-inducing strains, which infect primary macrophage and T-cell cultures. CXCR4 has been shown to be the principal co-receptor for primary syncytium-inducing HIV isolates (Feng *et al.*, 1996; McKnight *et al.*, 1997), while

CCR5 is the main co-receptor for non-syncytium-inducing isolates (Alkhatib *et al.*, 1996). Thus, HIV entry is inhibited in the presence of the chemokine ligands for these receptors: RANTES, MIP-1α and MIP-1β for CCR5 and SDF-1 for CXCR4 (Cocchi *et al.*, 1995; Bleul *et al.*, 1996; Oberlin *et al.*, 1996). The finding that CCR5 appears important for transmission while CXCR4-using viruses develop late in infection, make both these receptors important targets for anti-viral therapies (Huang *et al.*, 1996; Connor *et al.*, 1997). However, there is evidence to suggest that targeting the structure and folding of the GPCR using polypeptides derived from transmembrane domains may be a more effective strategy than inhibiting ligand binding (Tarasova *et al.*, 1999).

1.8. Viral GPCRs

Many of the herpesviruses encode homologues of cellular GPCRs that have probably been acquired from the host cell genome, and conserved because they confer a selective advantage. Various hypotheses exist as to the function of viral GPCRs but it is generally accepted that they play a role in virus persistence. Since the gammaherpesvirus GPCRs bear greatest resemblance to a mammalian chemokine receptor, it is possible that they have an immunomodulatory role and contribute to immune-evasion. Alternatively, their capacity to transduce signals and transform cells indicates a role in cell growth and proliferation, presumably to increase the number of infected cells. A third possibility is that they may function as homing receptors, trafficking infected cells to different lymphoid compartments. However, as cellular GPCRs participate in such a wide range of biological processes, it is perhaps unlikely that viral GPCRs share a single function. Table 1.8.1 lists examples of viral GPCRs and their homology with CXCR2 and the MHV-68 GPCR.

1.8.1. The KSHV GPCR

KSHV encodes a functional G-protein coupled receptor with homology to the mammalian IL-8 receptor, CXCR2 (Cesarman *et al.*, 1996b; Guo *et al.*, 1997). Competitive binding studies have shown that unlike CXCR2, the viral receptor binds a wide range of both CC and CXC chemokines. The KSHV GPCR exhibits agonist-independent signalling activity, as measured by the accumulation of the second

Table 1.8.1. Similarity between Viral GPCRs and Mammalian CXCR2

Virus	GCR Homologue	αα-Identity with CXCR2	αα-Identity with MHV-68 ORF74
HVS	ORF74	30%	23%
KSHV	ORF74	27%	25%
RRV	ORF74	29%	27%
EHV-2	ORF74	21%	22%
EHV-2	E1	34%	23%
AHV-1	ORF10	21%	25%
EBV	EBI1 (Induced cellular gene)	40%	15%
CMV	US28	34%	28%
CMV	UL33	26%	23%
MCMV	M33	23%	21%
RCMV	R33	26%	19%
HHV-6	U12	19%	19%
HHV-7	U12	19%	23%

The percentage amino acid (αα) identity between each known herpesvirus GPCR homologues and the MHV-68 GPCR was calculated using the gcg10 "GAP" alignment programme. The identity between each viral receptor and CXCR2, the closest homologue of the MHV-68 GPCR, is also shown.

messenger, inositol phosphate (Arvanitakis *et al.*, 1997). This has also been demonstrated by deletion of the N-terminus, which abrogates chemokine binding but does not affect basal signalling activity (Ho *et al.*, 1999). However, signalling activity is up-regulated by the angiogenic chemokines IL-8 and growth-related protein α (GRO-α) and inhibited by angiostatic chemokines - interferon inducible protein (IP-10), platelet factor 4 (PF-4), monokine induced by IFNγ (MIG) and interferon-inducible T cell α attractant (I-TAC, GerasRaaka *et al.*, 1998; Gershengorn *et al.*, 1998). The GPCR signals via the phosphoinositide–protein kinase C pathway but also activates two other protein kinase pathways (Arvanitakis *et al.*, 1997; Bais *et al.*, 1998).

The KSHV GPCR has been shown to have a transforming activity when expressed in NIH3T3 cells and transformed cells expressing the GPCR are also tumorigenic in nude mice. The GPCR triggers an "angiogenic switch" resulting in secretion of large amounts of vascular endothelial growth factor (VEGF, Bais *et al.*, 1998). As KS is a vascular tumour, stimulation of angiogenesis by induction of VEGF and other angiogenic chemokines is likely to contribute to oncogenesis. *In situ* hybridisation experiments have detected expression of the GPCR in only a minority of spindle cells within KS lesions and in uninduced BCBL-1 cells, coincident with other lytic cycle genes (Kirshner *et al.*, 1999).

1.8.2. EBV and Cellular GPCRs

The Epstein Barr virus does not encode a GPCR homologue. However, it does induce expression of three GPCRs: EBI1, a homologue of CXCR2 (40% amino acid identity); EBI2, which shares limited amino acid identity (24%) with the thrombin receptor and BLR1 (Kaiser *et al.*, 1993; Birkenbach *et al.*, 1993; Burgstahler *et al.*, 1995; Dobner *et al.*, 1992). EBI1, now designated CCR7, is expressed exclusively in lymphoid tissues and activates B and T cells (Yoshida *et al.*, 1997). It is strongly up regulated in EBV-infected cells and is transactivated by EBNA-2. BLR1 is expressed in lymphocytes and is the first GPCR shown to be involved in B-cell trafficking. In transgenic mice lacking BLR1, lymphocytes fail to migrate into splenic follicles and since activated B cells do not traffic from the T-cell rich zone

into B-cell follicles, there are no functional germinal centres (Forster *et al.*, 1996). The advantage of BLR1 to EBV, a B-cell tropic virus, would presumably involve targeting of infected B cells to germinal centres for expansion. Interestingly, EBI1 is also induced by HHV-6 and HHV-7 (Hasegawa *et al.*, 1994).

1.8.3. The HVS GPCR

Like KSHV and MHV-68, the HVS ORF74 encodes a GPCR also known as ECRF3 (Nicholas *et al.*, 1992). The HVS GPCR selectively binds and responds to CXC chemokines, as measured by calcium fluxes in *Xenopus laevis* oocytes reconstituted with ECRF3 cRNA (Ahuja & Murphy, 1993). Unlike the KSHV GPCR, the signalling activity does not appear to be ligand-independent.

1.8.4. The CMV GPCRs

Four GPCR homologues have been identified in the HCMV genome: US27, US28, UL33 and UL78 (Chee *et al.*, 1990; Gompels *et al.*, 1995). US28 mediates vascular smooth muscle cell migration in response to CC chemokines, as evidenced by abrogation of this function in recombinant HCMV lacking US28 (Streblow *et al.*, 1999; Vieira *et al.*, 1998). It is likely that US28 plays a role in the pathology of vascular conditions associated with HCMV such as atherosclerosis and organ transplant vascular sclerosis (Melnick *et al.*, 1996; Peterson *et al.*, 1980; Zhou *et al.*, 1996). Human immunodeficiency virus (HIV-1 and HIV-2) utilises as many as ten different GPCRs as co-receptors for entry into cells, of which one is US28 (Pleskoff *et al.*, 1997).

An unusual feature of UL33 is its presence in the virus envelope, suggesting that it might be inserted directly into the host cell membrane upon virus entry (Margulies *et al.*, 1996; Welch *et al.*, 1991). Ablation of the mouse CMV (MCMV) UL33 homologue results in a lower mortality rate in immunocompromised rats and failure of the virus to replicate in the salivary glands (DavisPoynter *et al.*, 1997). A similar observation has been made of the UL33 gene in rat CMV (Beisser *et al.*, 1998). GPCR homologues are also present in the other human betaherpesviruses, HHV6 and

HHV-7 (Gompels *et al.*, 1995; Nicholas, 1996). HHV-6 UL12 encodes a functional receptor that binds CC chemokines (Isegawa *et al.*, 1998).

1.8.6. Other GPCR Homologues

The swinepox and capripox viruses both encode GPCR homologues, the function of which has not been determined (Cao *et al.*, 1995; Massung *et al.*, 1993). Other gammaherpesvirus GPCRs include the AHV-1 GPCR, rhesus rhadinovirus (RRV) ORF74 and equine herpesvirus 2 (EHV-2) E1 and E6 (Alexander *et al.*, 2000; Ensser *et al.*, 1997; Telford *et al.*, 1995). Again, little is known of the gene function although an *in vitro* transforming activity has been demonstrated for the RRV GPCR (Estep & Wong, 2000).

1.9. Viruses and Transformation

The study of DNA tumour viruses has provided a wealth of information regarding cellular transformation. Many tumour viruses and carcinogens will rapidly and reproducibly alter fibroblasts *in vitro* so that certain aspects of their morphology and growth characteristics come to resemble tumour cells. After being altered in this way, a cell is said to be transformed. Since the oncogenic properties of viruses, carcinogens and individual genes are conventionally tested on fibroblasts, transformation is defined by a set of differences in the growth properties of fibroblastic cells in culture. Transformed cells may exhibit any of the following characteristic changes, although a cell that possesses any one of these alterations does not inevitably possess all of the others (Tooze, 1973).

1.9.1. Fibroblastic Cells in Culture

Following explantation of human foetal cells, the type of cell that predominates in the primary culture is fibroblastic. Cultured "fibroblasts" have the same morphology as tissue fibroblasts but are less differentiated, retaining the capacity to form mesodermal cells such as fat, connective tissue and muscle if supplied with appropriate stimulatory factors. A primary human fibroblast culture will undergo approximately 50 rounds of cell division before they cease growth and die (Hayflick & Morehead, 1961). A lineage of cells originating from an initial explant has a finite

lifespan and is known as a cell strain. Unlike human cells, embryonic rodent cells readily give rise to cell lines, which are immortal. After about 30 divisions, a primary rodent cell strain will undergo "crisis", during which most of the cells die. At this time, a rare cell variant may emerge that continues to divide and takes over the culture. The emergent immortal cell line usually has an abnormal number of chromosomes and other changes in growth properties associated with transformation.

However, there is a clear distinction between immortalisation and transformation. Todaro and Green discovered that if a primary culture of mouse fibroblastic cells was maintained at low density throughout the crisis period, the post-crisis cell line retained normal growth controls and was not tumorigenic *in vivo* (Todaro, 1963). This cell line, named 3T3 because it was derived from $3x10^5$ cells *t*ransferred every 3 days, has an infinite capacity to divide but ceases division once the cells have formed a confluent monolayer. It was found that the degree of saturation density under which the cell lines were maintained was directly proportional to their tumorigenicity. Thus 3T12 was highly tumorigenic, requiring fewer cells and a shorter period of time to generate a tumour than 3T6 (Aaronson & Todaro, 1968). This demonstrated for the first time, the importance of cell-cell contact in the control of cell division. Despite retaining most of the features of untransformed fibroblasts, 3T3 cells are nevertheless aneuploid, immortal and are more readily agglutinated by lectins such as concavalin A (Stoker, 1967).

1.9.2. Properties of Transformed Cells

The growth of primary fibroblast cells is regulated by contact from neighbouring cells and it has been shown that cell contact plays a role in inhibiting cell division (Dulbecco, 1970). Primary fibroblast cells and the cells of most fibroblastic cell lines also require attachment to a solid surface in order to divide, a phenomenon termed anchorage dependence of multiplication (Stoker *et al.*, 1968). In transformed cells, these growth control signals are de-regulated resulting in loss of contact inhibition and anchorage-independent growth.

Most mammalian cells in tissue culture require to be supplemented with serum in order to divide (Jainchill, 1970). Serum contains essential growth factors such as epidermal growth factor, insulin, insulinlike growth factor and transferrin, which are required by most cells in tissue culture (Gospodarowicz & Moran, 1976). The addition of fresh serum to a quiescent culture reinitiates growth and generates a number of cells proportional to the level of serum added, thus revealing that cell growth is controlled by factors other than cell-cell contact (Holley & Kiernan, 1968). Transformed cells, however, have a reduced serum requirement, presumably because overriding normal growth control mechanisms reduces the need for growth factors. In some cases, transformed cells produce both growth factors and their cognate receptors thus providing autocrine stimulation. Transformed cells are said to have lost the capacity for growth arrest and will even continue dividing to the point at which the whole culture dies because nutrients in the medium have been exhausted.

Many of the changes in growth properties of transformed cells are related to alterations in cell surface molecules. Modification of the links between cytoskeletal elements and proteins in the plasma membrane are thought to cause the increased mobility of the latter. This would account for the greater ease of agglutination by antibodies or lectins in transformed cells than in normal cells (Burger & Martin, 1972). Actin microfilaments that normally extend the length of normal cells tend to be either diffusely distributed or concentrated at the cell surface of transformed cells (Weber, 1974). In addition to altering the mobility of plasma membrane proteins, changes in the cytoskeleton also directly affect cellular morphology, resulting in a more rounded appearance. Transformed cells also exhibit a higher glycolytic activity due to the cell surface expression of a rapid high-affinity glucose transporter that is ordinarily expressed on brain cells and erythrocytes (Hatanaka, 1974). The amount of fibronectin on the surface of transformed cells is also greatly reduced, which is presumably a major factor in facilitating anchorage independent growth. Transformed cells often secrete plasminogen activator, an enzyme that converts plasminogen to the protease, plasmin. The secretion of proteases by transformed cells has been implicated in their capacity to form tumours as protease digestion of the basal lamina may promote tumour cell invasion of surrounding tissues.

Another feature of transformed cells is genetic instability. Most cell lines are aneuploid, for example 3T3 cells have on average 70 chromosomes per cell but their chromosomal complement continually expands and contracts in culture. Tumour cells are generally heteroploid but may also exhibit chromosomal translocations e.g. Burkitt's lymphoma cells. Of course, all the changes associated with immortalisation and transformation described above have a genetic basis. A change in the expression of one or two genes, proto-oncogenes or tumour suppressors, is sufficient to transform a cell. The "two-hit hypothesis" of oncogenesis was proposed by Knudson, who showed that both alleles of the *Rb* gene must be inactivated in order to produce retinoblastoma (Knudson, 1971). Transfection of primary rat embryo cells with the *ras* oncogene induces morphological changes associated with transformation but does not result in immortalisation (Land *et al.*, 1986). However, co-transfection with *ras* and *myc* produces fully transformed, immortal, tumorigenic cell lines, thus indicating that there are two broad classes of oncogene: cytoplasmic *ras*-like oncogenes and nuclear *myc*-like oncogenes. Although this model of co-operativity has been complicated by experiments showing single-step transformation with certain oncogenes, it suggests that immortal cell lines such as 3T3 provide a good substrate for assessing transformation since they already possess an active *myc*-like oncogene. Many types of proto-oncogene have been described including growth factors and their receptors, intracellular signalling molecules, transcription factors and cell-cycle control genes. In general, oncogenes are mutated or aberrantly expressed forms of cellular genes. Oncogenic viral genes are often homologues of cellular genes but some viruses encode transforming genes that have no cellular counterparts. Examples of herpesvirus oncogenes are given in the following sections.

1.9.3. Viral Oncogenes

The role of viral oncogenes is somewhat puzzling as tumour development has been described as a biological "dead-end" for the virus, killing the host and with it any chance of transmission to a new host (Moore & Chang, 1998). Promoting cell division facilitates viral replication and transmission to daughter cells but is an inefficient method of generating progeny. It seems more likely that viral oncogenes

have a more complex function, perhaps subverting tumour-suppressor pathways in order to overcome host defences against infection and induce lytic cycle replication. It is also worth remembering that tumours associated with viruses usually occur in organisms other than the natural host. For example herpesvirus saimiri and adenoviruses are only oncogenic in non-natural host species (Rangan *et al.*, 1977; Wold *et al.*, 1994). Herpesviruses in general are tumorigenic only in abnormal circumstances such as immunosuppression of the host e.g. AIDS-KS or the expression of a complementing oncogene e.g. Burkitt's lymphoma. Viruses have accrued a range of strategies to escape cell cycle control and these include the acquirement of cellular gene homologues involved in cell growth control as well as a number of unique genes with oncogenic properties.

1.9.4. Cyclin D Homologues

HVS, KSHV and MHV-68 all encode cyclin homologues (Cesarman *et al.*, 1996b; Nicholas *et al.*, 1992; Virgin *et al.*, 1997). Cyclins activate cellular kinases (cyclin-dependent kinases, cdks), which in turn phosphorylate cell cycle checkpoint molecules and thereby facilitate progression of cells through the cell cycle. Activation of cdk6 results in phosphorylation of the retinoblastoma protein (RB1), causing it to dissociate from the replication factor, E2F. The KSHV v-cyclin activates cdk6, a kinase normally stimulated by type D cyclins that drive the cell through the G_1 checkpoint. The KSHV v-cyclin is more promiscuous than cellular type D cyclins, not only promoting phosphorylation of RB1 but also histone H1. (Chang *et al.*, 1996; Godden-Kent *et al.*, 1997). The MHV-68 v-cyclin also promotes cell cycle progression and transgenic mice that express the gene in T cells develop lymphomas (van Dyk *et al.*, 2000). In addition, recombinant MHV-68 lacking the v-cyclin gene does not reactivate efficiently from latency (Hoge *et al.*, 2000). Therefore, viral cyclins are likely to play an important role overriding cell growth control mechanisms to facilitate virus replication and persistence.

1.9.5. Anti-Apoptotic Gene Homologues

In a normal cell, the main defence against de-regulated growth and uncontrolled division is induction of apoptosis, a strategy of self-destruction for the greater benefit

of the whole organism. Apoptosis is also a typical response to virus infection, thereby limiting production of virus progeny. Viruses have evolved a variety of mechanisms to inhibit apoptosis, one of which appears to be encoding a homologue of the cell-rescue factor, Bcl-2. The *bcl*-2 gene was first discovered at the junction of a chromosomal translocation in B cell lymphomas. Overexpression of *bcl*-2 permits survival of B cells that would normally undergo programmed cell death, thus resulting in lymphoma. Functional Bcl-2 homologues are encoded by HVS, KSHV, EBV and MHV-68 (Henderson *et al.*, 1993; Nava *et al.*, 1997; Sarid *et al.*, 1997; Roy *et al.*, 2000; Wang *et al.*, 1999). Therefore, conservation of a functional Bcl-2 homologue in gammaherpesviruses suggests that overcoming apoptosis is an important part of their pathobiology.

The KSHV vIRF has a transforming activity *in vitro* and effectively inhibits IFN signalling (Gao *et al.*, 1997; Zimring *et al.*, 1998). This appears to be a common characteristic among viruses: adenovirus E1A protein and the EBV EBNA-2 protein both down regulate the IFN response (Kanda *et al.*, 1992; Reich *et al.*, 1988). As mentioned previously, IFN is an important part of the antiviral immune response. For instance, IFN up-regulates MHC molecules to enhance immune detection and shuts off nucleic acid synthesis in uninfected cells. In addition, the IFN response initiates cell cycle arrest and apoptosis in infected cells. Therefore, inhibiting IFN signalling is another mechanism viruses have evolved for suppressing apoptosis that also has an oncogenic consequence.

KSHV also encodes a homologue of the cellular FLICE-inhibitory protein (FLIP), a molecule that blocks cell death induced by the tumour necrosis (TNF) family of receptors. A likely function of the KSHV v-FLIP is to protect virus-infected cells from apoptosis and therefore contribute to persistence (Thome *et al.*, 1997). Although no direct transforming activity of v-FLIP has been demonstrated *in vitro*, there is evidence that overriding apoptotic signals mediated by the TNF family of receptors (i.e. CD95) is a contributory factor in the development of hepatomas and melanomas (Hahne *et al.*, 1996; Strand *et al.*, 1996).

1.9.6. Gammaherpesvirus-specific Oncogenes

A feature of the HVS, EBV, KSHV and RRV genomes is the presence of oncogenic genes at the left-hand end. These are all transmembrane signalling proteins and include HVS saimiri transforming protein (STP) and tyrosine kinase interacting protein (Tip); EBV LMP-1; KSHV K1 and K15; RRV R1.

HVS STP is essential for the transforming activity of the virus and variations in STP differentiate the three HVS strains. Strain C is the most highly oncogenic and the association of STP-C with the cellular oncogene, *ras*, is vital to its transforming activity (Jung & Desrosiers, 1995). STP-C also interacts with the tumour necrosis associated factor (TRAF) family of signalling molecules, which activate the cellular transcription factor, NFκB. Interestingly, STP-A does not interact with Ras and although it associates with TRAFs, it fails to activate NFκB (Lee *et al.*, 1999). HVS Tip, which is unique to strain C, associates with the T-cell tyrosine kinase, Lck, thereby inhibiting signalling via the T cell receptor. Mutations in Tip that abrogate interaction with Lck increase the transforming activity of strain C (C488), which suggests that Tip may have a role in regulating the transforming potential of HVS (Duboise *et al.*, 1998b).

The EBV LMP-1 protein is an integral membrane phosphoprotein with a cytoplasmic amino terminus, six transmembrane domains and a long cytoplasmic carboxy terminus. Multimerisation of LMP-1 mimics the activated ligand-induced conformation of the B cell receptor, CD40. This generates a constitutive activation signal that contributes to B cell transformation. The carboxy terminus of LMP-1 has been shown to interact with members of the TRAF signalling proteins and also the TNF-receptor death domain (TRADD) (Eliopoulos *et al.*, 1999; Mosialos *et al.*, 1995). This results in activation of the transcription factor, NFκB, and the JNK signalling pathway (Huen *et al.*, 1995), which induces expression of a variety of proteins including epidermal growth factor (EGFR, Miller *et al.*, 1995b) and Bcl-2. The TRAF-binding domain has been shown to be essential for transformation (Wang *et al.*, 1988).

KSHV K1 and RRV R1 are positional homologues of HVS STP and EBV LMP-1. K1 and R1 both have transforming activity in rodent fibroblasts and can functionally substitute HVS STP in T cell transformation (Damania *et al.*, 1999; Lee *et al.*, 1998). K1 is a constitutively active receptor that interacts with cellular tyrosine kinases such as Vav, p85 and Syk to activate the transcription factor, NFAT, leading to cell growth (Lagunoff *et al.*, 1999).

1.9.7. SV40 Tumour Antigens

Although not a herpesvirus, the transforming activity of the SV40 early region is mentioned here with relevance to the transformation assays described in chapter three. The SV40 early region encodes three tumour antigens (TAg): large T, middle T and small T, all arising from differential splicing of the early region transcript. Although large TAg is sufficient to cause immortalisation and transformation of primary rodent cells, small TAg enhances large TAg-induced transformation (Bikel *et al.*, 1987). The mechanism of large TAg transformation appears to be functional inactivation of Rb by preventing binding of the replication factor, E2F (Sullivan *et al.*, 2000).

Therefore, viruses have evolved various ways of circumventing the host antiviral responses, including apoptosis, immune activation and cell cycle arrest. Either by encoding viral homologues of cellular signalling molecules or by inducing expression of host proteins, gammaherpesviruses are capable of modulating host cell growth and proliferation to facilitate virus replication and persistence.

AIMS

The aim of this project was to characterise the MHV-68 GPCR and investigate its role in the pathogenesis of the virus. Several approaches were taken including analysis of the transcription pattern of the GPCR gene and the sub-cellular localisation of the GPCR protein. The potential transforming activity of the gene was examined and lastly, attempts were made to define the contribution of the gene to MHV-68 pathogenesis by constructing a recombinant virus lacking the GPCR.

Chapter 2: Materials and Methods

2.1. General Solutions

Unless otherwise stated, all chemicals were obtained from Sigma or BDH Merck. Manufacturers are listed in appendix 4.

TE buffer:	10mM Tris-HCl [pH 7.9]
	1mM EDTA [pH 8.0]
TAE buffer:	0.04M Tris-acetate
	1mM EDTA [pH 8.0]
TBE buffer:	0.045M Tris-borate
	1mM EDTA [pH 8.0]
L-Broth (LB):	10g/L bactotryptone
	5g/L bacto yeast extract
	10g/L NaCl
LB Agar:	LB + 15g/L bacto-agar
SOC medium:	LB + 10mM glucose, 10mM $MgSO_4$, 20mM $MgCl_2$
Glucose solution:	50mM glucose
	25mM Tris-HCl [pH 8.0]
	10mM EDTA
Denaturation solution:	0.2M NaOH
	1% SDS (w/v)
Neutralisation solution:	3M potassium acetate
	2M acetic acid [pH 4.8]
20x SSPE:	3M NaCl
	0.2M $NaH_2PO_4.H_2O$
	0.02M EDTA [pH 7.4]
20x SSC:	3M NaCl
	0.3M sodium citrate
50x Denhardt's solution:	1% Ficoll (w/v)
	1% PVP (w/v)
	1% BSA (w/v)
TBS:	50mM Tris
	0.15M NaCl

PBS: 137mM NaCl
2.7mM KCl
4.3mM Na_2HPO_4
1.4mM KH_2PO_4

dH_2O: Distilled water was used throughout for making up solutions and for rinsing glassware etc. Milli-Q water (Millipore) was used in all enzymatic reactions and in preparation of nucleic acid and protein.

2.2. DNA Extraction and Manipulation

Molecular cloning was carried out essentially as described by (Sambrook *et al.*, 1989).

2.2.1. Plasmid Vectors

The following plasmid vectors were used: pKS(-) (Stratagene), pGEX-2T (Amersham Pharmacia), pBABE/*puro* (Morgenstern & Land, 1990) and pVR1255*luc*$^-$ (Hartikka *et al.*, 1996). Appendix 2 contains diagrammatic information on these plasmids.

2.2.2. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was typically performed in a volume of 100µl containing a final concentration of 0.25mM 2'-deoxyadenosine-5'-triphosphate (dATP), 2'-deoxyguanosine-5'-triphosphate (dGTP), 2'-deoxycytidine-5'-triphosphate (dCTP), 2'-deoxythymidine-5'-triphosphate (TTP, Ultrapure dNTP set, Amersham Pharmacia), reaction buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl [pH 8.3], Life Technologies) 0.2pmole/µl each primer, 100-500ng DNA template and 1U *Taq* DNA polymerase (Life Technologies). PCR was carried out in thin-walled 0.5ml Eppendorf tubes on an Omnigene thermal cycler (both Hybaid) under the following conditions: reactions were heated to 94°C for 5mins, then held at the annealing temperature while the enzyme was added ("hot-start"), thus limiting non-specific amplification. The block was programmed to repeat 25-40 cycles of 30s denaturation at 94°C, 1min annealing at specific temperatures, 1.5-3min extension at

72°C ending with 5mins at 72°C. DNA was also amplified using *PfuTurbo*™ DNA polymerase (Stratagene). Reactions were performed in a volume of 100µl containing cloned *Pfu* DNA polymerase reaction buffer (20mM Tris-HCl [pH 8.8], 2mM $MgSO_4$, 10mM KCl, 10mM $(NH_4)_2SO_4$, 0.1% Triton® X-100, 0.1mg/ml nuclease-free BSA, Stratagene) 0.25mM each dATP, dCTP, dGTP, TTP, 0.3pmole/µl each primer, 100-500ng DNA template and 2.5U *Pfu* Turbo™ DNA polymerase. The cycling parameters were similar to those used for PCR with *Taq* DNA polymerase except that no "hot-start" was required. Oligonucleotide sequences and annealing temperatures are listed in Appendix 1. All primers were obtained from MWG-Biotech with the exception of primer pair 1 (Cruachem). PCR products were analysed by agarose gel electrophoresis.

2.2.3. Agarose Gel Electrophoresis

DNA (typically $^1/_{10}$ reaction or ≤1µg) was mixed with 1µl gel loading buffer (15% ficoll type 400 (w/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 0.25% orange G (w/v) in dH_2O). Sample volumes were equalised with dH_2O and loaded onto agarose gels containing 0.8-2% agarose (SeaKem®, Flowgen) and 0.5µg/ml (w/v) ethidium bromide in TAE buffer. Electrophoresis was carried out in a horizontal tank (BIO-RAD) containing TAE or TBE buffer at 60-70V. The size of DNA bands was estimated by comparison with DNA molecular weight markers (1kb DNA ladder, Life Technologies). DNA bands were visualised using a short wave UV transilluminator (UVP inc.).

2.2.4. Isolation of DNA Fragments from Agarose Gels

DNA bands were excised from agarose gels using a scalpel blade. Dialysis tubing was prepared by boiling for several minutes in 1mM EDTA. The tubing was washed thoroughly in dH_2O and closed at one end with a clip. The agarose gel slice was inserted into the tubing with 0.5-1.0ml TAE. All air bubbles were removed and the tubing was sealed at the other end. The dialysis tubing was placed in a mini-gel tank and electrophoresed at 60V for one hour. The current was briefly reversed (30s) to remove any DNA attached to the inside of the tube. Elution of DNA was verified using a short wave UV transilluminator. The DNA was transferred to a clean

Eppendorf tube and a phenol-chloroform extraction was performed. The DNA was recovered by ethanol precipitation.

2.2.5. Purification of PCR Products

Amplified DNA was purified using the Wizard® PCR Product Purification kit (Promega) according to the manufacturer's instructions. Briefly, DNA was resuspended in 100µl direct purification buffer (50mM KCl, 10mM Tris-HCl [pH 8.8], 1.5mM $MgCl_2$, 0.1% Triton® X-100) then incubated with 1ml purification resin for 1min. During this time, the slurry was mixed three times using a vortex. The mixture was applied to a mini-column using a 5ml syringe. The column was washed with 2ml 80% propan-2-ol and dried by centrifugation (10,000*g*, 2mins). The DNA was eluted with 50µl MilliQ dH_2O at 70°C.

2.2.6. Phenol-Chloroform Extraction

DNA was purified by a phenol-chloroform extraction procedure. A mixture of phenol [pH 7.9], equilibrated with TE, chloroform and iso-amyl alcohol in a ratio of 25:24:1 (v/v) was added to an equal volume of DNA in a 1.5ml Eppendorf tube. The contents of the tube were thoroughly mixed using a vortex for 30s then centrifuged (9000*g*) for 2mins. The upper aqueous phase containing the DNA was transferred to a clean tube and the procedure was repeated until the interface between the aqueous and non-aqueous solutions became clear. A single chloroform extraction was performed to ensure removal of phenol from the DNA. The DNA was recovered by ethanol precipitation.

2.2.7. Ethanol Precipitation

DNA was precipitated in 2.5vols ethanol (96% Analar) and 0.5vol 7.5M ammonium acetate or 0.1vol 3M sodium acetate at -20°C for one hour. The precipitated DNA was centrifuged (9000*g*, 10mins), washed in 70% ethanol and resuspended in an appropriate volume of TE or MilliQ dH_2O.

2.2.8. Quantification of DNA

DNA concentration was assessed using a fluorometer (DyNAQUANT™ 200, Hoefer Amersham Pharmacia). Briefly, DNA was diluted 1:1000 in a solution containing Hoechst dye (bisbenzimide). Binding of the dye to the minor groove of DNA results in a shift in the emission spectrum that can be measured using a filtered photodetector. The DNA concentration is calculated by comparing the signal to that obtained with a DNA standard of known concentration.

2.2.9. Digestion of DNA with Restriction Enzymes

DNA restriction enzymes were obtained from Life Technologies and used with the supplied buffers according to the manufacturer's instructions. DNA restrictions were typically carried out for three hours at 37°C in a volume of 100µl containing 10 units of each enzyme per 1-10µg of DNA.

2.2.10. Blunt-Ending of DNA Fragments

DNA overhanging ends generated by restriction enzymes were filled in to facilitate ligation with blunt-ended vector DNA. DNA fragments with 3'-overhanging ends (100-500ng) were incubated in a volume of 100µl containing a final concentration of 0.25mM dATP, dGTP, dCTP, TTP, T4 DNA polymerase buffer (33mM tris-acetate [pH 7.9], 65mM sodium acetate, 10mM magnesium acetate, 5mM dithiothreitol, 100µg/ml bovine serum albumin) and 1U T4 DNA polymerase (Life Technologies). The mixture was incubated at 37°C for 20mins.

2.2.11. De-phosphorylation of Linearised DNA

In order to prevent re-circularisation during ligation reactions, the 5'-phosphate group on each end of linearised vector DNA was removed using calf intestinal phosphatase (CIP, Roche). Following restriction digest, the DNA was incubated with 10U CIP for 20mins at 37°C. The DNA was extracted with phenol-chloroform, ethanol-precipitated and quantified prior to ligation.

2.2.12. Ligation of DNA Fragments

Ligation was carried out overnight at 4°C in a final volume of 20µl containing approximately 250-500ng of the DNA fragment to be inserted and 50-100ng of linearised vector DNA in a 5:1 ratio, ligation buffer (50mM Tris-HCl [pH 7.6], 10mM $MgCl_2$, 1mM ATP, 1mM dithiothreitol, 5% (w/v) polyethylene glycol-8000) and 1U T4 DNA ligase (both Life Technologies).

2.2.13. Transformation of Competent Bacteria

Escherichia coli bacteria (Epicurean coli XL1-Blue sub-cloning-grade competent cells, Stratagene) were transformed according to Hanahan, 1983. Competent cells (50µl) were thawed on ice and incubated for 10mins with 0.8µl 1.44M β-mercaptoethanol in a 15ml polypropylene Falcon tube. Ligation mix (2µl) containing 30-60ng DNA was added and the mixture incubated on ice for a further 30mins. The bacteria were placed at 42°C for 45s and then chilled on ice for a further 2mins. SOC medium (900µl) was added and shaken for one hour at 37°C. Bacteria (25µl or 100µl) were spread onto LB agar plates containing an appropriate antibiotic (100µg/ml ampicillin or 30µg/ml kanamycin A, both Sigma). The remaining volume was centrifuged (9000*g*, 1min) and the pellet resuspended in 100µl before spreading onto a separate LB agar plate. Plates were incubated overnight at 37°C.

2.2.14. Small-Scale Preparation of Plasmid DNA

A number of single bacterial colonies (12-24) were grown overnight at 37°C with shaking in 10ml L-Broth containing 100µg/ml ampicillin or 30µg/ml kanamycin A. Bacteria were pelleted by centrifugation (1500*g*, 5mins) and resuspended in 200µl glucose solution containing 70µg/ml lysozyme (Sigma) and incubated at RT for 5mins. Bacteria were disrupted by the addition of 400µl denaturation solution, inverted several times to mix and incubated on ice for 5mins. Ice-cold neutralisation solution (300µl) was added to the tubes, which were mixed by inversion and placed on ice for 5mins. The mixture was centrifuged (11,000*g*, 5mins) and the supernatant transferred to a new Eppendorf containing 500µl ice-cold propan-2-ol. The

precipitated DNA was centrifuged (11,000*g*, 5mins) and resuspended in 200µl TE. Insoluble material was precipitated by the addition of 0.5vol 7.5M NH_4Ac. Following centrifugation, the supernatant containing the DNA was transferred to a new Eppendorf containing 2vols ice-cold ethanol and incubated at -20°C for one hour. The DNA was centrifuged (11,000*g*, 10mins), and the pellet washed with 70% ethanol, air-dried and resuspended in 50µl TE.

2.2.15. Small-Scale Preparation of Plasmid DNA for Sequencing

Plasmid DNA required for the purpose of sequencing was prepared using the QIAprep® Spin Miniprep Kit (QIAGEN), according to the manufacturer's instructions. A single colony was used to inoculate 5ml of LB (supplemented with an appropriate antibiotic) and grown overnight at 37°C with shaking. Bacteria were pelleted by centrifugation (1500*g*, 5mins) and resuspended in 250µl of buffer P1. The cells were lysed by the addition of 250µl of buffer P2 and then neutralised by the addition of 350µl of buffer N3. The cell debris was pelleted by centrifugation (10000*g*, 10mins) and the supernatant decanted onto the spin column. The column was centrifuged (10000*g*, 1min) and the flow-through was discarded. The column was washed by the addition of 750µl of Buffer PE and centrifuged as above. The flow-through was again discarded and the column centrifuged for an additional 1min to remove residual wash buffer. The DNA was eluted from the column by the addition of 50µl of buffer EB.

2.2.16. Large-Scale Preparation of Plasmid DNA on CsCl Gradients

Large-scale plasmid purification was carried out on CsCl gradients. Bacterial cultures (400ml) were grown overnight in LB containing appropriate antibiotic. Bacteria were centrifuged in 250ml centrifuge bottles (5000*g*, 5mins, 4°C) and the pellet resuspended in 20ml glucose solution containing 70µg/ml lysozyme. Following 10mins incubation at RT, the bacteria were disrupted by the addition of 40ml denaturation solution and chilled on ice for 5mins. A volume (20ml) of ice-cold neutralisation solution was added and the lysate incubated on ice for a further 15mins. The lysate was centrifuged (12,000*g*, 30min, 4°C) and the supernatant filtered through gauze into a clean bottle. DNA was precipitated in 0.6vol propan-2-

ol and pelleted by centrifugation as above after 30mins. The pellet was air-dried before being resuspended in 6ml TE and transferred to a 30ml corex tube. A volume (3ml) of 7.5M ammonium acetate was added and the mixture incubated on ice for 30mins. Following centrifugation (12,000*g*, 10mins, 4°C) the supernatant was transferred to a clean tube containing 2.5vols ethanol and incubated at -20°C for one hour. The DNA precipitate was pelleted by centrifugation (12,000*g*, 10mins, 4°C) and resuspended in 30ml 1:1 CsCl:TE (w/v) containing 140µg/ml ethidium bromide. The solution was transferred to plastic quick-seal centrifuge tubes (Beckman) and centrifuged for 16 hours at 200,000*g* using a Beckman Vti50 rotor. Two bands representing closed circular plasmid DNA were visualised using a UV light source. The lower band containing supercoiled plasmid DNA was removed by piercing the side of the tube with a hypodermic syringe. The whole band was taken off in a volume of less than 3ml. Ethidium bromide was extracted by repeated mixing with an equal volume of 1-butanol saturated with 1:1 CsCl:TE (w/v) until the upper phase became clear. The DNA was precipitated in 2.5vols ethanol at -20°C for one hour, pelleted by centrifugation (12,000*g*, 10mins) and washed in 70% ethanol. The DNA was resuspended in 10ml TE and dialysed against TE to remove the CsCl. The dialysed DNA was re-precipitated in ethanol and resuspended in 1ml MilliQ dH_2O.

2.2.17. Large-Scale Plasmid Preparation on Anion Exchange Columns

Large-scale plasmid purification was also performed using the CONCERT™ High Purity Plasmid Purification System according to the manufacturer's instructions (Life Technologies). The column was equilibrated with 30ml Equilibration Buffer (600mM NaCl, 100mM sodium acetate [pH 5.0], 0.15% Triton® X-100 [v/v]) and drained by gravity flow. An overnight culture (500ml) was centrifuged (5000*g*, 5mins) and the pellet resuspended in 10ml Cell Suspension Buffer (50mM Tris-HCl [pH 8.0], 10mM EDTA) containing 0.1mg/ml RNaseA. Bacteria were disrupted by the addition of 10ml Cell Lysis Solution (200mM NaOH, 1% SDS [w/v]) and incubated for 5mins at RT. The mixture was incubated for a further 5mins following addition of 10ml Neutralisation Buffer (3.1M potassium acetate [pH 5.5]). The mixture was then centrifuged (15,000*g*, 10mins) and the supernatant loaded onto the column. Once the solution in the column had drained by gravity flow, 60ml Wash

Buffer (800mM NaCl, 100mM sodium acetate [pH 5.0]) was applied to the column. The DNA was eluted by adding 15ml Elution Buffer (1.25M NaCl, 100mM Tris-HCl [pH 8.5]) and precipitated by the addition of 10.5ml isopropanol to the eluate. The mixture was centrifuged (15,000*g*, 30mins, 4°C) and the DNA pellet washed with 5ml 70% ethanol. Once dried, the pellet was resuspended in 500µl TE.

2.2.18. Preparation of DNA from Eukaryotic Cells

Small-scale preparation of DNA from cells was carried out using the QIAmp®DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, cells ($1x10^5$-$1x10^7$) were harvested and counted as described below (2.4.2). The cell pellet was resuspended in 200µl PBS and transferred to a 1.5ml Eppendorf tube containing 20µl proteinase K. The cells were mixed thoroughly with 200µl buffer AL and incubated at 56°C for 10mins. The lysate was mixed with 200µl 96% ethanol and applied to the spin column. The column was centrifuged (6000*g*, 1min) and the flow-through discarded. The column was washed first with 500µl buffer AW1 then 500µl buffer AW2. The DNA was eluted in 200µl buffer AE.

2.2.19. Large-Scale Preparation of Recombinant Virus DNA

BHK-21 cells ($8x10^6$) were infected with recombinant virus and grown in T175 flasks for five days or until 100% cytopathic effect was observed. The cells were scraped into 10ml of sterile PBS, pelleted (450*g*, 5mins) and resuspended in 10ml extraction buffer (0.1M EDTA, 0.5% SDS, 0.2M Tris-HCl [pH 8.0]) in polypropylene Falcon tubes. The cellular lysate was incubated with 20µg/ml RNase A at 37°C for 1 hour. Proteinase K (100 µg/ml) was added to the lysate and incubated at 56°C overnight. The DNA was subjected to three rounds of phenol-chloroform extraction followed by one chloroform extraction. The final supernatant was precipitated in 2vols 96% ethanol and 0.1vol 3M sodium acetate at RT. The DNA was washed in 70% ethanol and resuspended in 200-400µl TE. For Southern analysis, the DNA was quantified and three aliquots were each digested with a single restriction enzyme overnight. The DNA underwent phenol-chloroform extraction and re-precipitation prior to electrophoresis.

2.2.20. Southern Analysis of DNA

DNA was electrophoresed on a 0.8%-1% agarose gel. The DNA was denatured by soaking the gel in denaturation buffer (0.5M NaOH, 1M NaCl) for 40mins. The gel was then immersed in neutralisation buffer (0.5M Tris-HCl [pH 7.5], 1.5M NaCl) for 40mins. The gel was rinsed in dH_2O and equilibrated in transfer buffer (10xSSC) before assembly of the blotting rig. A piece of 3MM filter paper (Whatman) was pre-wetted with 10xSSC and used to cover the gel platform of an electrophoresis tank so that the ends were submerged in the10xSSC filling the wells of the tank. The gel was placed on top of the filter paper. A piece of membrane (0.22μM nylon membrane Magnagraph, MSI) was cut to exactly the same size as the gel, re-hydrated in dH_2O, and equilibrated in 10xSSC. The membrane was placed directly on top of the gel, ensuring that air bubbles were eliminated. The gel was surrounded with saran wrap to prevent the transfer buffer by-passing the gel into the stack of paper towels. Three pieces of 3MM filter paper, also cut to the same size, were equilibrated in 10xSSC and placed on top of the membrane. Dry paper towels formed the final layer and facilitated capillary transfer of DNA to the membrane. A weight was placed on top of the paper towels and the DNA transferred overnight. The following morning, the blot was carefully dismantled and the positions of the wells of the gel were marked on the membrane. The DNA was fixed to the membrane by UV cross-linking (Stratalinker 1800, Stratagene). The section of the blot corresponding to the DNA markers was carefully removed for staining using a scalpel blade. The membrane was either transferred to pre-hybridisation solution or stored at -20°C.

2.2.21. Staining of DNA Molecular Weight Markers

The membrane strip bearing the DNA markers was soaked in 1M acetic acid (10mins, RT). The markers were then transferred to staining solution (0.4M acetic acid, 0.4M sodium acetate, 0.2% methylene blue) and incubated for 10mins. The staining solution was poured off and the membrane washed in dH_2O. The distance each DNA band had migrated from the well was measured and plotted against the $\log_{10}$ of its molecular weight. A line was drawn through the points and the graph used to estimate the molecular weight of DNA bands detected by hybridisation.

2.2.22. Radiolabelling of DNA

DNA probes for hybridisation were labelled using the Random Primed DNA Labelling kit according to the manufacturer's instructions (Roche). Briefly, 25ng DNA was denatured by heating to 95°C for 10mins, cooled on ice and added to a solution containing 0.025mM dATP, TTP, dGTP, hexanucleotide primer, 50μCi [α^{32}P]dCTP (Amersham Pharmacia) and 2U Klenow polymerase (large fragment of DNA polymerase I). DNA was labelled for 40mins at 37°C and unincorporated nucleotides were removed by chromatography on a Nick® Sephadex G-50 column (Amersham Pharmacia). Labelled DNA was incubated with 0.1vol 1M NaOH for 5mins at 37°C immediately prior to hybridisation.

2.2.23. Hybridisation of Southern Blots with DNA Probes

The blot was pre-hybridised in 25ml hybridisation solution (6xSSC, 5% milk) for at least 2 hours at 68°C. The solution was poured off and replaced with 25ml fresh hybridisation solution containing the denatured DNA probe. The blot was incubated with the probe at 68°C overnight. The blot was washed for 15mins twice in each of the following solutions: 1xSSC, 0.1% SDS at RT; 1xSSC, 1% SDS at 37°C; 0.1xSSC, 1% SDS at 60°C. The blot was then wrapped in saran wrap and exposed to autoradiographic film (X-OMAT, Kodak) at -70°C. Films were developed using an X-OMAT automatic X-ray film processor.

2.2.24. Automated Sequencing of DNA

DNA sequencing reactions were performed using a SequiTherm EXCEL™ II DNA Sequencing kit (cycle sequencing protocol) on a 4000L automated sequencing machine (MWG-Biotech) by Ian Bennet (Dept. of Veterinary Pathology, University of Edinburgh).

2.2.25. Sequence Analysis of DNA

Storage of sequence, mapping of restriction sites and comparison to other database sequences was made using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package v.10 (Devereux *et al.*, 1984).

2.3. RNA Extraction and Manipulation

In all procedures involving RNA, precautions were taken to prevent degradation by RNase enzymes. Glassware and other equipment was routinely rinsed with 5M NaOH followed by copious rinsing with sterile dH_2O. All solutions were made up with chemicals reserved solely for RNA use. Barrier tips and sterile plastic-ware were used throughout. RNA samples and Northern blots were stored at -70°C.

2.3.1. Cytoplasmic RNA Extraction

Cells were washed twice in sterile PBS. Adherent cells were removed from the monolayer by scraping and centrifuged (450*g*, 5mins). The pellet was resuspended in 1ml ice-cold Tris-saline (25mM tris [pH 7.4], 130mM NaCl, 5mM KCl) and transferred to a 1.5ml Eppendorf tube. The addition of 100µl Igepal buffer (1% Igepal (Sigma)/ 0.5% sodium deoxycholate/ 0.01% dextran sulphate in Tris-saline) resulted in lysis of plasma membranes but not nuclear membranes. The lysate was centrifuged (2000*g*, 1min) and the nuclear pellet discarded. The supernatant was transferred to a new tube containing 500µl (25:24:1) phenol:chloroform:iso-amyl alcohol (v/v), 25µl 20% SDS and 15µl 5M NaCl. After vigorous mixing using a vortex, the tube was centrifuged (11,000*g*) and the upper aqueous phase removed to a clean tube. The RNA underwent three phenol-chloroform extractions, a single chloroform extraction and was recovered by ethanol precipitation. The RNA was centrifuged (11,000*g*, 20mins, 4°C), washed in 80% ethanol, air-dried and resuspended in 50µl sterile dH_2O (Sigma). RNA was quantified using a Cecil spectrophotometer. The concentration was calculated using the following equation where an A_{260} of 1.0 is equivalent to 40mg/ml of RNA:

RNA concentration = Absorption at 260nm (A_{260}) × 40 × dilution factor

The purity of the nucleic acid sample, with respect to protein contamination, was assessed using the ratio A_{260}/A_{280}, where a value greater than 1.8 indicates that the sample is free from protein contamination. The RNA was also analysed by gel electrophoresis on a 1% agarose gel containing TBE.

2.3.2. Purification of Polyadenylated RNA from Total RNA

Total RNA was enriched to 50% polyadenylated RNA using a kit (mRNA Purification kit, Amersham Pharmacia). RNA was diluted in 1ml elution buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA), denatured at 65°C for 5mins and placed on ice. A volume (200µl) of sample buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA, 3M NaCl) was added to the RNA and the mixture applied to the column. The column was centrifuged (350*g*, 2mins, Beckman TJ-6). The column was washed and centrifuged twice with 250µl high salt buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA, 0.5M NaCl), three times with 250µl low salt buffer (10mM Tris-HCl [pH 7.4], 1mM EDTA, 0.1M NaCl) and eluted in 1ml elution buffer pre-warmed to 65°C. The mRNA was precipitated in 2.5vols ethanol containing 100µl sample buffer and 10µl glycogen. The precipitate was recovered by centrifuging for 10mins at 4°C (12,000*g*), washed in 80% ethanol, air-dried and resuspended in 10µl MilliQ dH_2O.

2.3.3. Purification of Total RNA

Total RNA was also isolated from eukaryotic cells using the RNeasy® Mini Kit, QIAGEN. Briefly, cells ($1x10^7$) were harvested, pelleted by centrifugation (450*g*, 5mins) and lysed by the addition of 600µl buffer RLT. The lysate was homogenised by passing through a 20-G needle fitted to a syringe. Ethanol (70%, 600µl) was added and the sample was applied to the mini spin column. Following brief centrifugation, the column was washed once with 700µl buffer RW1 and twice with 500µl buffer RPE. The RNA was eluted in 30-50µl RNase-free dH_2O.

2.3.4. Direct Purification of Polyadenylated RNA from Cells

Polyadenylated RNA was also purified directly from cells using the QuikPrep™ Micro mRNA Purification kit (Amersham Pharmacia). Cells ($1x10^7$) were harvested, counted and transferred to a 1.5ml Eppendorf. The cells were pelleted and resuspended in 400µl extraction buffer containing guanidium thiocyanate and N-lauroyl sarcosine and thoroughly homogenised using a vortex. The sample was diluted in 800µl elution buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA) and centrifuged (11,000*g*, 1min) to produce a cleared cellular homogenate. Oligo(dT)-

cellulose (1ml of 25mg/ml solution) was centrifuged simultaneously and the buffer discarded. The cleared homogenate was added to the oligo(dT)-cellulose and gently mixed by inversion for 3mins to allow binding of the polyadenylated RNA. The sample was centrifuged (11,000*g*, 10secs) and the pellet was washed with 1ml high-salt buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 0.5M NaCl). Five washes were performed with the high-salt buffer followed by two washes with low-salt buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 0.1M NaCl). The pelleted oligo(dT)-cellulose was resuspended in 300µl low-salt buffer and applied to a spin column. The column was centrifuged (11,000*g*, 5secs) and washed three times with 500µl low-salt buffer. The polyadenylated RNA was eluted with 200µl elution buffer pre-warmed to 65°C. The elution step was repeated a second time resulting in a final volume of 400µl. The RNA was ethanol precipitated in 10µl glycogen solution (5-10mg/ml glycogen), 40µl 2.5M potassium acetate [pH 5.0] and 2.5vols 96% ethanol at -70°C for one hour. The RNA was centrifuged, washed and quantified as described above.

2.3.5. Northern Analysis

RNA was electrophoresed on a 1.2% agarose gel containing 1x MOPS buffer and 2.2M formaldehyde. RNA samples were diluted in 0.5x MOPS containing 17.5% formaldehyde and 50% formamide in a volume of 20µl. Typically, 1-3µg of polyadenylated RNA or 5-10µg total RNA was loaded per well. RNA molecular weight markers (5µg, 0.24-9.5kb RNA ladder, Life Technologies) were prepared in the same way. RNA was denatured at 65°C for 15mins, cooled on ice and mixed with 2µl loading buffer (50% glycerol, 1mM EDTA [pH 8.0], 0.25% bromophenol blue) immediately prior to loading. The gel was electrophoresed overnight in 1xMOPS and the buffer re-circulated using a pump. The gel was rinsed in dH_2O before being transferred to the blotting rig. The Northern blot was assembled identically to the Southern blot (2.2.20) with the exception of the transfer buffer, which was 10xSSPE. As with the Southern blot, transfer took place overnight after which the blot was carefully removed, the positions of the wells marked and the RNA fixed by UV cross-linking. The lane corresponding to the RNA molecular weight markers was removed and stained in the same way as the DNA markers.

2.3.6. Radiolabelling of RNA

In vitro transcription of RNA was carried out using a kit (Riboprobe *In Vitro* Transcription Systems, Promega) according to the manufacturer's instructions. The plasmid DNA template (0.2-1.0μg) was linearised with an appropriate restriction enzyme as described above. Following digestion, the DNA was extracted with phenol-chloroform and precipitated in 96% ethanol. The pellet was resuspended in 1μl MilliQ dH_2O. The components of the transcription reaction were added in the following order in a final volume of 20μl: 5x transcription optimised buffer, 100mM DTT, 20U recombinant RNasin ribonuclease inhibitor, 2.5mM each rATP, rGTP, rCTP, 100μM rUTP, template DNA, 50μCi [α-^{32}P]rUTP (Amersham Pharmacia), 20U T7 RNA polymerase. The transcription mixture was incubated at 37°C for one hour. The DNA template was removed by digestion with RNAse-free DNAseI (1U/μg DNA) at 37°C for 15 minutes. The radiolabelled transcripts were isolated via phenol-chloroform extraction and ethanol precipitation. The final pellet was resuspended in 50μl TE and immediately added to the hybridisation mix.

2.3.7. Hybridisation of Northern Blots with DNA Probes

Northern blots were pre-hybridised for at least 2 hours at 45°C in a mixture containing 50% formamide, 5xSSPE, 5xDenhardt's, 0.5% SDS, 100μg/ml sheared single-stranded salmon sperm DNA. Hybridisation took place overnight at 45°C in a similar mixture containing also 10% dextran sulphate and 200μg/ml salmon sperm DNA. High stringency washes were carried out as follows: 2x15mins in 5xSSPE/ 0.5% SDS at RT; 2x15mins in 1xSSPE/ 0.5% SDS at 37°C; 3x15mins in 0.1xSSPE/ 1% SDS at 65°C. The blot was exposed to autoradiographic film (X-OMAT, Kodak) at -70°C or to a phosphorous-coated screen (Molecular Dynamics). The film was developed using an X-OMAT automatic developer (Kodak). The phosphorous-coated screens were visualised using a phosphorimager (Molecular Dynamics).

2.3.8. Hybridisation of Northern Blots with Riboprobes

The hybridisation conditions for RNA probes are similar to those described for DNA probes (2.3.7) except that the pre-hybridisation, hybridisation and washing solutions all contained 1% SDS.

2.3.9. Stripping of Northern Blots

DNA probes were removed from Northern blots by incubating in recently boiled dH_2O containing 0.1% SDS for 15mins. The blot was then washed in 6xSSC for 15mins at RT before being exposed to autoradiographic film to verify complete removal of the labelled probe.

2.3.10. Reverse Transcription PCR

RNA (2μg total RNA or 0.5-1μg polyadenylated RNA) was added to the following reaction mixture containing a final concentration of 1x first strand synthesis buffer (Life Technologies), 1mM each dATP, dCTP, dGTP, and TTP, 1pmole primer, 2mM DTT and 10U RNAse-free DNAseI (Roche) in a final volume of 25μl. The mixture was incubated at 37°C for 30mins to ensure complete digestion of any contaminating DNA. Heating to 80°C for 5mins inactivated the DNaseI. The reaction mixture was cooled on ice before addition of 200U reverse transcriptase (Superscript II $RNaseH^-$ Reverse Transcriptase). The cDNA synthesis reaction was carried out at 42°C for one hour. The enzyme was heat-inactivated at 95°C for 5mins and the tubes cooled on ice. Typically, 2μl of the reaction was used as a template in subsequent PCR reactions.

2.3.11. Rapid Amplification of cDNA Ends (RACE)

The polyadenylation site of the GPCR transcript was identified by 3'RACE. Total RNA was harvested from C127 cells infected with MHV-68 at 24h post-infection. Reverse transcription was carried out as described above using an oligo(dT) primer. The cDNA was amplified with a primer corresponding to the 5' region of GPCR and the oligo(dT) primer.

Attempts were made to identify the unknown 5'-end of the GPCR transcript using a 5'RACE kit (5'-Full RACE Core Set, Takara Shuzo) according to the manufacturer's instructions. The target RNA was the same as for 3'RACE. Synthesis of cDNA was carried out with a primer complementary to the 5' end of the GPCR coding region thus transcribing the unknown upstream region of the GPCR transcript. The target RNA was degraded by treating with RNAseH for one hour at 30°C and the cDNA recovered by ethanol precipitation. The cDNA was ligated with T4 RNA ligase overnight at 16°C, resulting in circularisation or concatomerisation of the cDNA. Primers were designed to the 5'-end known coding region of the GPCR. Nested PCR was carried out resulting in amplification of the unknown region lying between two copies of the known region.

2.4. Culture of Cells and Virus

2.4.1. Cell Lines

All cells lines were cultured in sterile plastic-ware (Nunc) and incubated at 37°C in equilibrium with humidified 5% CO_2.

CELL LINE	DESCRIPTION	REFERENCE
BHK-21	Hamster kidney fibroblast cells	(Stoker, 1961)
C127	RIII mouse mammary carcinoma cells	(Lowy, 1978)
NIH 3T3	Mouse fibroblast cells	(Bojan *et al.*, 1983)
293	Human embryonic kidney epithelial cells	(Graham *et al.*, 1977)
NS0	Non-immunoglobulin secreting mouse myeloma cells	(Kholer, 1976)
S11	Mouse B lymphoma cells carrying latent MHV-68	(Usherwood *et al.*, 1996c)
U937	Human Caucasian histiocytic lymphoma exhibiting monocytic characteristics	(Sundstrom & Nilsson, 1976)

Table 2.4.1. Description of cell lines

Baby Hamster Kidney (BHK-21) cells were cultured in Glasgow modified Eagle medium (GMEM, Life Technologies) supplemented with 10% (v/v) tryptose phosphate broth (Life Technologies) and 10% (v/v) new born calf serum (NBCS, Life Technologies), 70μg/ml penicillin (Merck BDH), 10μg/ml streptomycin

(Sigma) and 2mM L-glutamine (Merck BDH). Murine 3T3 cells were maintained in Dulbecco's Modified Eagles medium containing glutamax (DMEM, Life Technologies) and supplemented with 10% non-heat-treated NBCS, 70μg/ml penicillin and 10μg/ml streptomycin. The 293 cell line, was also maintained in DMEM supplemented with 10% FCS, 70μg/ml penicillin, 10μg/ml streptomycin and 2mM L-glutamine. The B cell lines (NS0 and S11) and the U937 line were cultured in RPMI 1640 (Life Technologies) containing 10% foetal calf serum (FCS, Globepharm), 70μg/ml penicillin, 10μg/ml streptomycin, 50μM β-mercaptoethanol (Sigma) and 2mM L-glutamine.

2.4.2. Harvesting and Counting of Cells

Adherent cells were maintained in sub-confluent growth and passaged by removing them from the monolayer with trypsin. The medium was poured off and the monolayer washed in 0.02% versene. The monolayer was then incubated with 0.25% trypsin (Life Technologies) until the cells could be removed from the surface of the flask by gentle tapping. The trypsin was then diluted in an equal volume of medium and cells were centrifuged (450*g*, 5mins). Non-adherent cells were also centrifuged for 5mins at 450*g*. The cell pellet was resuspended in appropriate growth medium and a small aliquot (50μl) was mixed with 50μl 0.1% trypan blue (w/v). The number of un-stained viable cells was counted using a haemocytometer.

2.4.3. Electroporation of Cells

Sub-confluent (60-80%) cells were washed and removed from the monolayer. Cells ($2x10^6$) were resuspended in 1ml medium and transferred to a chilled electroporation cuvette (EquiBio) containing 20μg of the DNA to be transfected. Cells were electroporated using a double-pulse setting (HV=600v, 25μF, 99Ω; LV=260V, 1500μF, 329Ω; 0.1s inter-pulse delay) on an Easyject electroporator (EquiBio). Cells were immediately transferred to a universal tube containing fresh medium then divided between two wells of a 6-well plate and incubated at 37°C. The efficiency of transfection was estimated using a control plasmid expressing GFP. Transiently transfected cells were generally harvested 24 hours post-infection.

2.4.4. Lipofection of Cells

Cells (60-80% confluent) were transfected using the DOTAP Liposomal Transfection Reagent (Roche). The DNA was diluted in Hepes buffer to a final concentration of 0.1μg/μl in a volume of 50μl. DOTAP (6μg per 1μg of DNA) was also diluted in Hepes buffer to a final volume of 100μl. The DNA was added to the DOTAP solution, gently mixed and incubated at room temperature for 10-15mins. The DOTAP/DNA mixture was added to fresh culture medium at a concentration not exceeding 30μg/ml DOTAP and applied gently to the cells using a pipette. Cells were incubated for 24 hours at 37°C.

2.4.5. Generation of Stably Transfected Cell Lines

Murine NIH 3T3 cells were transfected with the expression vector pBABE/*puro* encoding GPCR, EBV LMP-1 or empty plasmid. Post-transfection, 3T3 cells were incubated at 37°C for 3 days before being exposed to the selection agent (3μg/ml puromycin, Sigma). The concentration of puromycin required to select only cells carrying the puromycin resistance gene was previously determined. Foci of resistant cells were identified and isolated using plastic rings coated in silicone. The focus of cells within the ring was removed with 100μl trypsin and immediately transferred to a 10mm well of a 24-well plate containing 1ml DMEM. Cells were propagated in 1.5 μg/ml puromycin.

2.4.6. Focus Formation Assay

Murine NIH 3T3 cells that were transiently or stably transfected with expression constructs encoding GPCR, EBV LMP1 or SV40 early region, were grown to confluence in 6 well-plates. The medium was replaced on alternate days for 3-4 weeks until cellular foci were observed under the light microscope.

2.4.7. Growth in Soft Agar

Soft agar assays were performed as described by Baichwal & Sugden, 1988. A 3.5% stock solution of low gelling temperature agarose (SeaPlaque®, Flowgen) was prepared in sterile PBS. The stock solution was diluted 1:7 in medium equilibrated to 37°C. Six-well plates were layered with 2ml of the resulting 0.5% agarose and

placed at 4°C to set. Cells ($1x10^4$) were resuspended in 1.8ml medium warmed to 37°C. Cells were then mixed with 0.2ml 3.5% agarose and layered on top of the 0.5% agarose. The agarose was gelled at 4°C for several minutes before being incubated at 37°C. The cells were supplemented with 2-3 drops of medium (DMEM + glutamax, Life Technologies) containing 10% FCS, 70μg/ml penicillin and 10μg/ml streptomycin, on alternate days for 3 weeks. Cellular foci (diameter >50μM) were counted with the aid of a calibrated graticule and a light microscope.

2.4.8. Tumorigenicity Assay

The tumorigenicity of stably transfected cell lines expressing the GPCR was tested in nude mice (Bantin and Kingman). Six groups of four six-week old mice each received a subcutaneous injection in the left flank of $1x10^6$ cells in a volume of 100μl. The mice were monitored for the formation of tumours at the site of injection. The experiment was repeated using $1x10^7$ cells. Each mouse received the same cell line as in the previous experiment but injected in the right flank.

2.4.9. Preparation of Virus Stocks

Stocks of MHV-68 were generated in BHK-21 cells. Cells were grown in T175cm^2 flasks (~$1x10^7$ cells/flask) and harvested and counted as described. Cells were resuspended in medium ($1x10^7$/ml) and infected with MHV-68 at a multiplicity of infection (MOI) of 0.01. The cells were incubated for one hour at 37°C with shaking and seeded ($2x10^6$ cells/flask) in T175cm^2 flasks. The infected cells were incubated at 37°C for 5-7 days until gross cytopathic effect was observed. The cells were dislodged from the monolayer by scraping or gentle tapping of the flask and centrifuged (2000*g*, 20mins, 4°C). The pellet was resuspended in a small volume of sterile PBS (5-10ml) and homogenised with 20-30 strokes of a chilled Wheaton-Dounce homogeniser. The homogenate was transferred to a glass universal tube and sonicated in a water-bath for 15mins at 4°C. Following centrifugation (2000*g*, 20mins, 4°C), the supernatant was transferred to a clean universal tube and kept on ice. The pellet was resuspended in 1ml sterile PBS, re-homogenised and centrifuged (2000*g*, 20mins, 4°C). The supernatants were pooled, aliquoted and stored at -70°C.

2.4.10. Titration of MHV-68

Titres of virus stocks were determined using a plaque assay as described by Sunil-Chandra *et al.*, 1992a. Duplicate serial dilutions of the virus sample were prepared (10^{-1} to 10^{-8}) in 1.8ml medium. BHK-21 cells ($5x10^5$) were added to each virus dilution and incubated for one hour at 37°C with shaking. The infected cells were transferred to 60mm culture plates and incubated at 37°C for four days. Uninfected BHK-21 cells were also seeded in duplicate plates at the same density as a negative control. Cells were fixed in 4% buffered formaldehyde (v/v) (Surgipath) and stained with 0.1% toluidine blue. Virus plaques were counted using a light microscope (Leitz ortholux) thus allowing calculation of virus titre in plaque forming units (pfu/ml).

2.4.11. Infective Centre Assay

The titre of virus in latently infected splenocytes or B cell lines was determined using the infective centre assay. For splenocytes, the spleen was removed from the mouse and transferred to a petri dish containing a few mls of medium. The splenocytes were teased from their outer coating using a scalpel blade and transferred to a pre-rinsed sterile universal tube. Following centrifugation (450*g*, 5mins), the supernatant was decanted and the cell pellet resuspended in the remaining few drops of medium. The erythrocytes were lysed by addition of 1ml sterile dH_2O for 15s. The osmotic balance was immediately restored by the addition of 9ml PBS and once the cellular debris had settled, the cell suspension was carefully removed. The cells were centrifuged as before, resuspended in 5ml RPMI and counted. Duplicate serial dilutions (10^{-1}-10^{-3}) of the cells were prepared and added to 60mm plates containing $5x10^5$ freshly harvested BHK-21 cells in RPMI. The cells were incubated for five days at 37°C, fixed in 4% formal saline and stained in 0.1% toluidine blue. Virus plaques were counted using a light microscope. An aliquot of splenocytes was freeze-thawed to disrupt the cells and used for a plaque assay to determine the titre of free virus in the sample.

2.4.12. Generation of MHV-68 and MHV-76 Recombinants

Recombinant viruses were generated by co-transfection of BHK-21 cells with virus DNA and a homologous recombination cassette. The DNA sequences extending 2kb upstream and downstream of the GPCR coding region were amplified and inserted into the pKS(-) vector. The GFP gene, under control of the CMV promoter, was inserted between the two flanking sequences. The cassette was excised from pKS(-) prior to transfection. BHK-21 cells were transfected (see 2.4.3) with 10μg virus DNA and 10μg of the DNA cassette. Recombination of the cassette with virus DNA resulted in the generation of virus that expressed the GFP gene. Following transfection, the cells were transferred to 6-well plates and incubated at 37°C overnight. The following morning, the cells were washed twice with 2ml medium and overlaid with medium containing 1% agarose (Seaplaque®, Flowgen). The plates were incubated at 37°C and examined daily for the presence of GFP^+ virus plaques using a UV microscope (Nikon Diaphot 200).

2.4.13. Plaque Purification of Recombinant Virus

A plaque purification method was used to separate recombinant virus expressing GFP from wild-type virus. The positions of isolated GFP^+ plaques were marked on the underside of the plate. A 1ml Pasteur pipette was used to remove an agarose plug containing virus-infected cells. The agarose plug was transferred to a 1.5ml Eppendorf tube containing 500μl medium. Virus particles were released from cells by sonication at 4°C for 60s followed by three rounds of freeze-thawing at -70°C. The lysate was centrifuged for 2mins (11,000*g*) to remove cellular and agarose debris. A volume of the supernatant (100-250μl) was used to re-infect cells and the procedure was repeated until no wild-type plaques were observed. Subsequent plaque purification and liming dilution assays were carried out using C127 cells because the virus plaques formed on this cell line were more discreet than those formed on BHK-21 cell monolayers.

2.4.14. Limiting Dilution Assay

Virus samples obtained from picking plaques were serially diluted (1:5, 1:10, 1:20, 1:50, 1:100, 1:250, 1:500, 1:1000, 1:5000) to estimate the virus titre. C127 cells (1-

$2x10^4$) were grown in 5mm 96-well plates. Each dilution of virus was added to 10 wells containing a C127 monolayer and the number of recombinant and wild-type plaques formed was scored in each well. C127 cells were then infected with virus at a concentration of 0.4pfu/well giving rise to single plaques in 40% of wells. The single isolated plaques were harvested and the virus DNA analysed by Southern blotting.

2.4.15. Infection of Mice with Recombinant Virus

Transgenic mice lacking the α/β interferon receptor (Dutia *et al.*, 1999) were infected with a mixed population of wild type and recombinant MHV-76. Four mice were infected intranasally with either $1x10^2$ or $1x10^3$ pfu virus in a volume of 40µl sterile PBS.

2.4.16. Ligand Binding Assay

Cells (293, NIH3T3 or U937) were harvested, counted and washed in Hank's Balanced Salt Solution (HBSS). Cells ($1x10^5$) were incubated in 200µl HBSS containing 1mM PMSF and either 0.02pmole 2000Ci/mM human recombinant [^{125}I]IL-8 plus 20pmoles unlabelled IL-8, or [^{125}I]IL-8 alone (both Amersham Pharmacia). The samples were rotated at 4°C for two hours. The cells were centrifuged (450*g*, 2mins) and washed three times with 1ml HBSS. The final cell pellet was solubilised in 200µl 0.4M NaOH and a 100µl sample was mixed with scintillation fluid (Optiphase Hisafe, Wallac). The radioactive emission of each sample was measured in counts/min using a liquid scintillation counter (1450 Microbeta Plus, Wallac).

2.5. Immunological Methods

2.5.1. Production of a Fusion Protein

A 93bp fragment corresponding to the predicted N-terminal region of the GPCR was amplified by PCR. The product was inserted into the bacterial expression vector, pGEX-2T (Amersham Pharmacia) in frame with the *Schistosoma japonicum* glutathione-S-transferase gene (GST). Ligation and transformation were carried out

as described (2.12 and 2.13). Positive clones were tested for expression of the fusion protein. Bacterial colonies were picked and grown overnight in 10ml LB containing 100μg/ml ampicillin then diluted 1:10 in fresh LB. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 0.5mM during exponential growth phase ($O.D._{595}$ = 0.6). The bacteria were grown for a further three hours.

2.5.2. Purification of a Fusion Protein

Bacterial cultures were centrifuged (2000*g*, 5mins) and the pellet resuspended in 300μl PBS containing 1% (v/v) triton, 1mM PMSF and 1% (v/v) aprotinin. The bacteria were lysed by sonication and the insoluble material was pelleted by centrifugation (11,000*g*, 5mins). An aliquot of the insoluble fraction was retained for analysis and the soluble fraction was incubated for 5mins at RT with 50μl 50% glutathione sepharose beads (Amersham Pharmacia) that had been equilibrated with PBS. The beads were washed once in 1ml PBS + 1% Triton® and centrifuged at low speed (250*g*, 2mins). This was repeated twice using PBS alone. A volume of elution buffer (150μl) was added and the beads pelleted by centrifugation as before. Elution buffers were comprised of increasing concentrations of glutathione (5-15mM), NaCl (0.1M-3.0M) or urea (3.0M-5.0M). Both the eluate and glutathione-sepharose fraction were analysed by SDS-PAGE.

2.5.3. Generation of Antibody in Rabbits

Two NZW rabbits were immunised with fusion protein. Each rabbit received two intra-muscular injections in a volume of 100μl containing 25μl bed volume of the fusion protein-sepharose complex resuspended in 25μl PSB and mixed with 50μl adjuvant (TiterMax® Gold, Sigma). A blood sample (5-10ml) was taken from each animal prior to immunisation and three weeks post-immunisation. Both rabbits received three booster injections at three-week intervals. Blood samples were taken three weeks after each injection and the serum assessed for reactivity with the GPCR by western blotting and immunoprecipitation.

2.5.4. Preparation of Serum

Blood samples were allowed to clot overnight at 4°C. The clot was detached from the sides of the universal tube using a Pasteur pipette prior to centrifugation (450*g*, 5mins). The serum supernatant was carefully removed and stored at 4°C.

2.5.5. Preparation of Peptides

Two peptides were synthesised corresponding to predicted hydrophilic regions of the GPCR (MWG Biotech) for the purpose of raising anti-GPCR antibody in sheep.

GPCR1: H_2N-**E**$_{17}$**N**$_{18}$**S**$_{19}$**S**$_{20}$**L**$_{21}$**S**$_{22}$**Y**$_{23}$**D**$_{24}$**D**$_{25}$**Y**$_{26}$**Y**$_{27}$**D**$_{28}$**N**$_{29}$**A**$_{30}$**T**$_{31}$**W**$_{32}$-COOH (16mer)

GPCR2: H_2N-**C**$_{178}$**A**$_{179}$**M**$_{180}$**E**$_{181}$**L**$_{182}$**G**$_{183}$**G**$_{184}$**T**$_{185}$**T**$_{186}$**R**$_{187}$**V**$_{188}$**S**$_{189}$**V**$_{190}$**P**$_{222}$**V**$_{223}$ **L**$_{224}$**I**$_{225}$**I**$_{226}$**I**$_{227}$**V**$_{228}$**M**$_{242}$**G**$_{243}$**K**$_{244}$**K**$_{245}$**Y**$_{246}$**R**$_{247}$**I**$_{248}$**Y**$_{249}$**V**$_{250}$**S**$_{251}$-COOH (30mer)

GPCR1 was conjugated via its amino terminus to bovine serum albumin (BSA) using the heterobifunctional reagent, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Kitagawa & Aikawa, 1976). BSA was dissolved in PBS at a concentration of 10mg/ml. A 25mg/ml solution of MBS was prepared in dimethylformamide and 0.1vol was added drop-wise to the BSA. The reaction was stirred for 30mins at RT. The activated BSA was separated from free MBS by gel filtration. A Sephadex® G-25M column (PD-10, Amersham Pharmacia) was equilibrated with 20ml PBS according to the manufacturer's instructions. Non-specific binding of the activated carrier was blocked by the addition of 2.5ml 10mg/ml BSA. The activated BSA (2.5ml) was eluted from the column in a volume of 3.5ml. The GPCR1 peptide (0.9mg) was resuspended in PBS and added to the activated BSA (0.35mg) in a ratio of 1 mole peptide to 50 amino acids BSA resulting in a 12-fold molar excess of peptide. The coupling reaction was carried out in a final volume of 90µl in a 0.5ml Eppendorf. The tube was rotated at RT for three hours. Conjugated peptide was stored at -70°C.

2.5.6. Generation of Antibody using Peptides

Two Finn sheep (8F042 and 8F045) were immunised with plasmid DNA encoding the GPCR. One sheep was also vaccinated with DNA encoding granulocyte macrophage colony stimulating factor (GMCSF). A volume of 250µl was injected into each leg muscle at the hip and shoulder. Sheep 8F045 was immunised with 4x 25µg of the pVR1255+GPCRHA3 construct in MilliQ dH_2O. Sheep 8F042 received 4x 25µg of the pVR1255+GPCRHA3 construct and 4x 25µg of pEGFP-N1+GMCSF construct (Craig Watkins, University of Edinburgh) in MilliQ dH_2O. A secondary DNA vaccination was administered after an interval of four weeks. Both sheep received a tertiary vaccination with 4x 12.5µg GPCR1 (conjugated to BSA carrier) and 4x 12.5µg GPCR2 resuspended in PBS 1:1 adjuvant (TiterMax® gold) in a volume of 4x 250µl. A blood sample was taken from each sheep 2 weeks after the peptide injection.

2.5.7. SDS-Polyacrylamide Gel Electrophoresis

Proteins were analysed by discontinuous polyacrylamide gel electrophoresis according to the method of Laemmli, 1970. Samples were diluted 1:1 in gel loading buffer (100mM Tris-HCl [pH 6.8], 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and denatured at 100°C for 5mins prior to loading. Electrophoresis was carried out on 15% resolving gel (15% v/v) acrylamide/methylene bisacrylamide (Protogel, National Diagnostics), 0.375M Tris-HCl [pH 8.8], 0.1% SDS. Stacking gel was 5% acrylamide, 0.125M Tris-HCl [pH 6.8], 0.1% SDS. Gels were set by addition of 2% ammonium persulphate and 0.08% N, N, N', N'-tetraethylmethylenediamine (TEMED, Sigma). SDS-PAGE running buffer was comprised of Tris-glycine (39mM glycine, 48mM Tris) and 0.5% SDS. Protein molecular weight was estimated by comparison with pre-stained broad-range standards (BIO-RAD). Gels were stained in Coomassie blue (0.25% Coomassie brilliant blue, 10% glacial acetic acid, 45% methanol) and de-stained in 20% methanol + 5% glacial acetic acid.

2.5.8. Western Analysis

Proteins were transferred to PVDF membrane (Immobilon-P, Millipore) in transfer buffer (1x Tris-glycine, 20% methanol). Non-specific binding to the membrane was blocked by incubation with blotto (5% Marvel milk in TBS) for 30mins at RT. The membrane was incubated with the primary antibody in 10-20ml blotto for 1-2 hours at a temperature and concentration recommended by the manufacturer. The membrane was washed three times in TBS + 0.2% Tween then incubated with the secondary antibody for 30mins. The blot was washed extensively in TBS + 0.2% Tween and TBS alone then developed in NBT/BCIP (Sigma FAST™ 5'Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium tablets).

2.5.9. Radiolabelling of Cells and Immunoprecipitation

BHK-21 cells were grown to 80% confluence in 6-well plates. The monolayer was infected with 5pfu/cell MHV-68 in a volume of 500µl for one hour at 37°C. The medium containing the virus was replaced with 2ml fresh medium. At 12 hours post-infection, the medium was removed and the monolayer washed in serum-free, methionine-free medium (Life technologies). The cells were incubated for four hours at 37°C in 3ml of the serum-free, methionine-free medium containing 1mCi ^{35}S-methionine. The medium was removed and the monolayer washed once with PBS. The cells were lysed by addition of 1ml RIPA buffer (20mM Tris [pH 7.2], 0.15M NaCl, 1% Triton® X-100, 1% sodium deoxycholate, 0.1% SDS), transferred to an Eppendorf tube. PMSF (1mM) and 5% aprotinin were added to the lysate and stored at -70°C.

The lysate was pre-cleared by incubating with 50% (v/v) sepharose 4B in RIPA buffer with rotation for four hours at 4°C. A volume of the test serum (20µl) was added to the lysate and incubated overnight at 4°C with rotation. As a positive control, an aliquot of lysate was incubated with 2.5µl polyclonal antibody to MHV-68. The pre-immune serum was used as a negative control. The lysate samples were incubated with 40µl 50% (v/v) protein A sepharose in RIPA buffer for four hours at 4°C with rotation. The protein A sepharose was centrifuged (450*g*, 5mins) and the pellet washed five times with RIPA buffer. The final pellet was resuspended in 40µl

SDS-PAGE sample buffer and boiled for 5mins. The sample was centrifuged (450*g*, 5mins) to remove the protein A sepharose before being loaded on an SDS-PAGE gel. Following electrophoresis, the gel was fixed in 10% glacial acetic acid and 30% methanol before being impregnated with EN^{3}HANCE™ (Dupont) for one hour at RT. The gel was then immersed in dH_2O for 30mins to precipitate the protein. The gel was dried and exposed to autoradiographic film.

2.5.10. Immunofluorescence

Cells ($2x10^5$) were washed in PBS, removed from the monolayer, centrifuged and resuspended in 100µl PBS. Cells were applied to glass microscope slides (Surgipath) using a Cytospin (Shandon). The slides were air-dried and fixed in acetone for 3mins at -20°C. Fixed cells were incubated with 100µl PBS containing 5% BSA (Sigma) at 37°C for 30mins to block non-specific binding. Cells were washed in PBS + 0.05% Tween for 5mins followed by PBS alone for 5mins before incubation with the primary antibody. The primary antibody was diluted in 100µl PBS at a concentration recommended by the manufacturer and incubated for 1 hour at RT or 37°C. Cells were washed three times in PBS + 0.05% Tween then incubated for 30mins with the secondary antibody. The cells were washed three times in PBS + 0.05% Tween and once in PBS alone. At this stage, cell nuclei were sometimes counterstained with propidium iodide. Cells were incubated with PBS containing 1ng/ml propidium iodide and 10ng/ml DNase-free RNaseA for 30mins at 37°C in darkness. Cells were mounted in Citifluor (UKC) and examined using a UV microscope (Nikon Diaphot 200 or Leica TCSNT confocal microscope).

2.5.11. Prediction of Protein Structure

Structural motifs were predicted from protein sequence using the following programmes that can be found at the listed web sites. The position of transmembrane helices and the orientation of the MHV-68 GPCR in the membrane was predicted using "TMpred" (Hofman, 1993) that is found at http://www.ch.embnet.org/software/TMPRED_form.html. The presence of common motifs was determined using "Scan Prosite" that is found at http://wwwexpasy.cbr.nrc.ca/tools/scnpsit1.html. The alignment of multiple proteins was performed using the Blosum

scoring matrix with the "ClustalW" programme (Thompson *et al.*, 1994) that is found at http://www.ch.embnet.org/software/ClustalW.html. The prediction of phosphorylation sites was made using the programme "NetPhos" (Blom *et al.*, 1999) that is found at http://www.cbs.dtu.dk/services/NetPhos/.

Chapter Three: Results

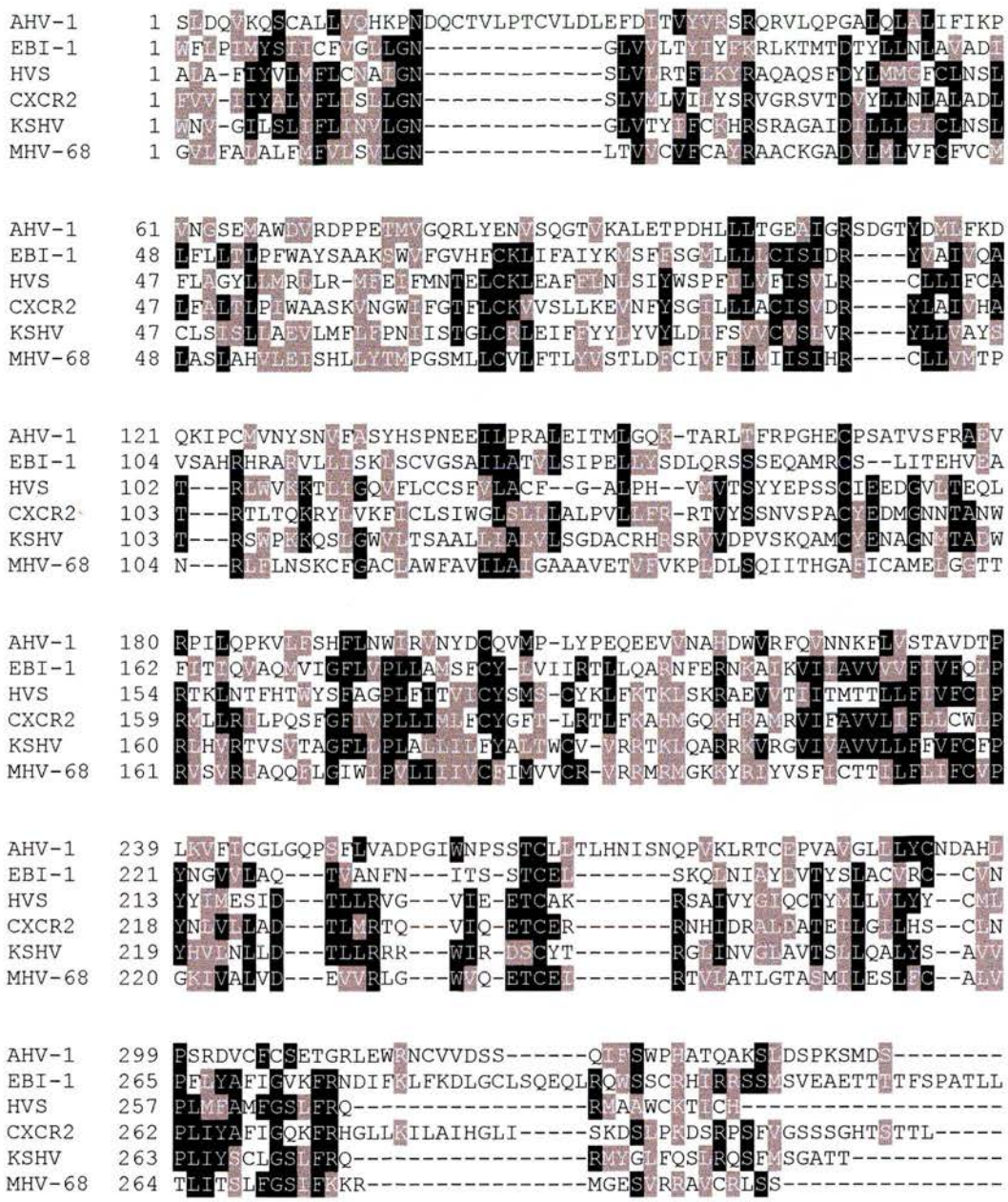
3.1. Sequence Analysis of the GPCR

Preliminary analysis of the MHV-68 DNA sequence revealed homology between MHV-68 ORF74 and the mammalian IL-8 receptor, CXCR2 (Virgin *et al.*, 1997). A database search was performed using the programme, "BLAST", which confirmed that the ORF74 gene product shared greatest identity with mammalian CXCR2 (28%) and the KSHV GPCR (25%) at the amino acid level. To demonstrate the level of amino acid identity between the MHV-68 GPCR and other gammaherpesvirus and mammalian GPCRs, the sequences were aligned using the programme, "Clustal W" (Thompson *et al.*, 1994). As shown in figure 3.1.1, the limited number of identical and similar amino acids (highlighted black and grey respectively) demonstrates that the similarity between the MHV-68 GPCR and the other gammaherpesvirus receptors is not high.

Since this level of amino acid identity was significant but low, it was necessary to investigate the relevance of the similarity by analysing the MHV-68 ORF74 sequence for conserved motifs that are characteristic of GPCRs using a variety of bioinformatics programmes that are available online (see section 2.5.11). As shown in figure 3.1.2A, analysis of potential transmembrane helices using the "TMpred" programme (Hofman, 1993) revealed the presence of seven 20-25 amino acid transmembrane regions, a feature of all GPCRs (highlighted yellow). This programme also showed that the orientation of the protein is consistent with other GPCRs: an extracellular hydrophilic amino terminal domain and an intracellular carboxy terminal domain (underlined). There are also predicted to be three intracellular (underlined) and three extracellular loops.

Most GPCRs possess a highly conserved aspartic acid-arginine-tyrosine (DRY) motif at the base of the third transmembrane domain and the start of the second intracellular loop that is required for coupling of G proteins (Dohlman *et al.*, 1991; Probst *et al.*, 1992). As shown in figure 3.1.2B, the MHV-68 ORF74 sequence contains a predicted histidine-arginine-cysteine (HRC) motif in this position (highlighted blue). The Y → C substitution occurs rarely in other GPCRs such as the *N*-formyl-L-leucyl-L-phenylalanine (fMLP) receptor and is therefore conservative

Figure 3.1.1. Alignment of Mammalian and Viral GPCRs



Sequence alignment of four gammaherpesvirus GPCRs (AHV-1 GPCR, HVS GPCR, KSHV GPCR, MHV-68 GPCR) with the mammalian IL-8 receptor (CXCR2) and the cellular chemokine receptor, CCR7, (EBI-1) which is induced by EBV. The sequences were aligned using the Blosum scoring matrix with the "Clustal W" programme, which can be found at http://www.ch.embnet.org/software/ClustalW.html. Residues that are identical in two or more sequences are highlighted in black, while synonymous residues are highlighted in grey.

Figure 3.1.2. Conserved Structural Motifs of the MHV-68 GPCR

A

```
  1   MLVLRDLDLE DLQAFLENSS LSYDDYYDNA TWIPDLASPC TVSLKYGVLF
 51   ALALFMFVLS VLGNLTVVCV FCAYRAACKG ADVLMLVFCF VCMLASLAHV
101   LEISHLLYTM PGSMLLCVLF TLYVSTLDFC IVFILMIISI HRCLLVMTPN
151   RLFLNSKCFG ACLAWFAVIL AIGAAAVETV FVKPLDLSQI ITHGAFICAM
                    *                              *
201   ELGGTTRVSV RLAQQFLGIW IPVLIIIVCF IMVVCRVRRM RMGKKYRIYV
251   SFICTTILFL IFCVPGKIVA LVDEVVRLGW VQETCEIRTV LATLGTASMI
301   LESLFCALVT LITSLFGSIF KKRMGESVRR AVCRLSS
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B

```
L A C I S V D R Y L A I V H A T R T   CXCR2
I G F I A L D R C I C V L H P V W A   fMLPR
V V C V S L V R Y L L V A Y S T R S   KSHV GPCR
L M I I S I H R C L L V M T P N R L   MHV-68 GPCR
```

Diagrammatic representation of the structural motifs predicted to be present in the MHV-68 ORF74 gene product. Panel A shows the amino acid sequence with predicted motifs as follows: transmembrane helices (highlighted yellow); N-linked glycosylation sites (boxed); intracellular domains (underlined); conserved residues (asterisk below); targets for phosphorylation (highlighted grey). B. Comparison of the sequence at the start of the 2nd intracellular loop in the mammalian CXCR2 and fMLP receptor, the KSHV GPCR and the MHV-68 GPCR. The sequences were aligned using the "Clustal W" programme. Residues that are identical in two or more sequences are highlighted black. The sequence surrounding the conserved arginine is boxed.

(Boulay *et al.*, 1990). However, the D → H change is a non-conservative mutation that has been observed in other gammaherpesvirus GPCRs and is associated with constitutive GPCR signalling (Ahuja & Murphy, 1993; Arvanitakis *et al.*, 1997; Burger *et al.*, 1999). The MHV-68 ORF74 gene product also contains two predicted N-linked glycosylation sites in the N-terminal domain (boxed) and cysteine and tryptophan residues (asterisked) that are all conserved in GPCRs. In addition, the third intracellular loop is highly cationic, which is a feature of mammalian chemokine receptors. Using the "NetPhos" programme (Blom *et al.*, 1999), two serine residues in the carboxy terminal domain (highlighted grey) were predicted to be potential targets for phosphorylation. When taken together, these predicted features indicated that the MHV-68 ORF74 gene product was most likely a functional receptor that had diverged from an ancestral mammalian chemoattractant receptor. The ORF74 product was therefore named MHV-68 GPCR.

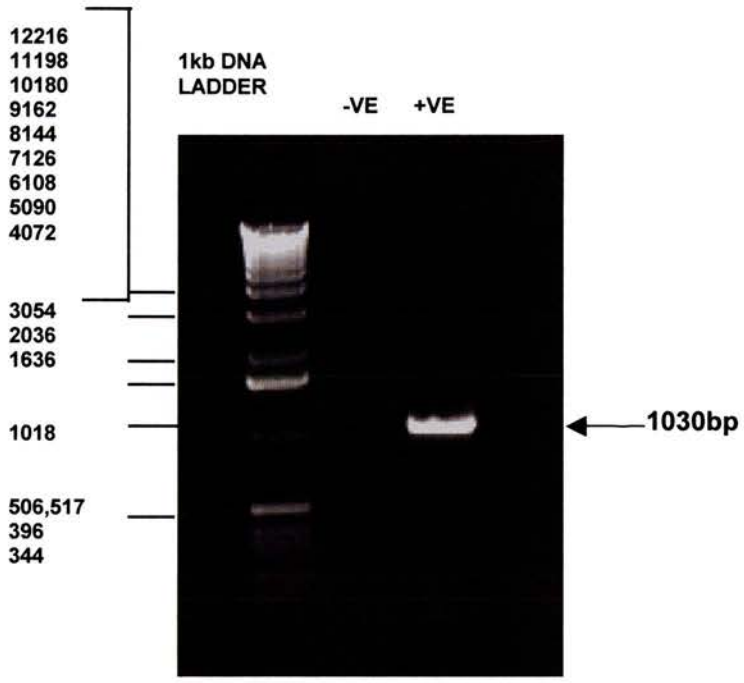
3.2. Transcription Pattern of the GPCR

3.2.1. Cloning of the MHV-68 GPCR

The aim of this project was to characterise the MHV-68 GPCR and examine its role in the pathogenesis of the virus. This was achieved, firstly, by establishing the transcriptional pattern of the gene and the sub-cellular localisation of the protein. A second approach was to investigate the potential transforming activity of the GPCR. Finally, attempts were made to generate a recombinant virus lacking the GPCR that would yield insight into the function of this gene *in vivo*.

It was essential to all these experimental strategies to amplify the GPCR DNA sequence. This allowed insertion of the gene into plasmid vectors and provided a DNA probe for hybridisation reactions. The coding region of the GPCR was amplified from MHV-68 genomic DNA using a standard PCR method (2.2.2). The primers (pair 1, appendix 1) included sites for the restriction enzymes, *Bam*HI and *Eco*RI at their 5' end which facilitated insertion of the 1030bp PCR product into the cloning vector, pKS (-) (appendix 2), using molecular cloning techniques. The PCR product is shown in figure 3.2.1.

Figure 3.2.1. PCR of the GPCR



The GPCR coding region was amplified from MHV-68 genomic DNA (100ng) using *Taq* polymerase. The PCR negative control (-VE) contained no DNA. The 1030bp PCR product was electrophoresed through a 0.8% agarose gel.

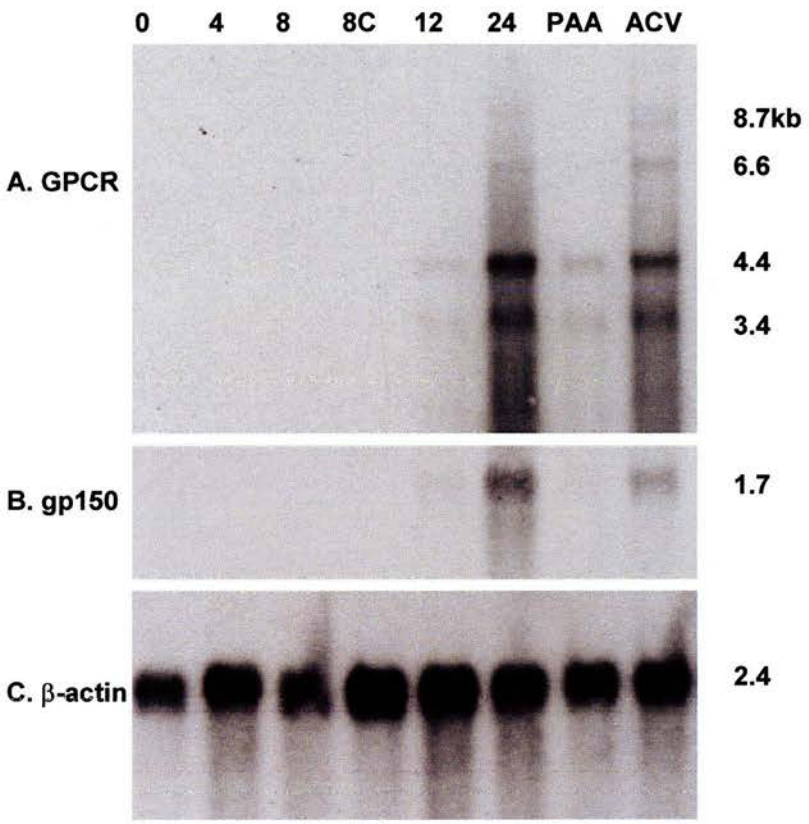
3.2.2. Northern Analysis

To understand the role of the GPCR in the virus life cycle, it was necessary to determine when the gene was transcribed. The temporal expression pattern of the GPCR during lytic infection was investigated by Northern blotting. BHK-21 cells ($8x10^7$) were infected with MHV-68 at a multiplicity of infection of 5 pfu/cell. Total RNA was harvested from the cells at 0, 4, 8, 12, and 24 hours post-infection. Chemical inhibitors were used to establish the kinetic class of gene expression: immediate early, early or late. Early and late gene expression requires *de novo* protein translation and thus can be suppressed using cycloheximide, a drug that inactivates the host cell ribosomes. Therefore, to isolate the RNA specific for the immediate early stage of infection, one flask of cells was treated with cycloheximide (100μg/ml) for 30mins prior to adsorption of the virus and harvested at 8 hours post-infection.

Late gene expression follows replication of the virus genome therefore drugs that inhibit virus replication prevent expression of late genes. Phosphonoacetic acid (PAA) inhibits DNA polymerase. Acyclovir (ACV) is a nucleoside analogue that is phosphorylated by the virus thymidine kinase but not by the cellular thymidine kinase. The pro-drug is activated only in infected cells and incorporated into the replicating DNA. The absence of a hydroxyl group prevents binding of the subsequent base resulting in premature chain termination. Therefore, early RNA was harvested (2.3.1) at 24 hours post-infection from cells that had been incubated with either PAA (100μg/ml) or ACV (100μm) for 2 hours prior to adsorption of the virus and throughout infection. Late RNA was obtained from un-treated infected cells at 24 hours post-infection.

Total RNA was enriched for polyadenylated RNA using a kit (Amersham Pharmacia, 2.3.2) and analysed by Northern blotting (2.3.5). The blot was hybridised (2.3.7) with a [α^{32}P]dCTP-labelled DNA probe corresponding to the GPCR coding region (2.2.22). Specific GPCR transcripts were detectable at 12 hours and 24 hours post-infection (Figure 3.2.2A). As a control for even loading and integrity of the RNA,

Figure 3.2.2. Northern Analysis of GPCR Transcription



Northern blot of mRNA (10μg/lane) harvested from BHK-21 cells infected with MHV-68 at 0, 4, 8, 12, and 24 hours post-infection. The Northern blot was hybridised with ^{32}P-labelled DNA probes corresponding to A. GPCR (3wk exposure), B. gp150 (24h exp) and C. β-actin (24h exp). C = cycloheximide ACV = acyclovir PAA = phosphonoacetic acid

the blot was stripped and re-hybridised with a DNA probe corresponding to the ubiquitous cellular gene, β-actin (Figure 3.2.2B). Lytic infection of the BHK-21 cells was confirmed by hybridisation of the blot with an MHV-68 structural gene, gp150 (figure 3.2.2C). Transcription of the GPCR was barely detectable using the Northern blotting technique. Blots of gels loaded with 5-10μg of total RNA or 1-3μg of polyadenylated RNA per lane did not produce a hybridisation signal. Expression of the GPCR was only detected when 10μg of polyadenylated RNA was loaded in each lane of the gel. Following hybridisation, the blot was exposed to a phosphorous-coated screen for 24 hours. Analysis of the screen using a phosphorimager revealed a faint signal. However, an exposure time of three weeks was required to detect a visible signal on autoradiographic film. Therefore, like the KSHV GPCR, the MHV-68 GPCR was expressed at very low abundance.

Transcription of the GPCR was not blocked by PAA, which indicates that the GPCR is an early gene. Expression appeared to be down regulated in the presence of PAA but this could have resulted from the toxicity of PAA to the BHK-21 cells. Transcription of the classic late gene, gp150, was completely abolished in the presence of PAA. ACV did not inhibit transcription of the GPCR, which again indicates that the GPCR is an early gene. However, there was low-level expression of gp150 in the presence of ACV, which suggested a certain degree of leakage through this block. Since the GPCR transcripts were not detectable at early time-points during lytic infection, it was not possible to determine whether their expression was inhibited by cycloheximide. At 8 hours post-infection, it is likely that the GPCR transcripts were expressed at a level beyond the sensitivity limits of this assay.

The size of each band was calculated using a graph generated from RNA molecular weight standards that were electrophoresed alongside the RNA samples. The $\log_{10}$ of molecular weight was plotted against the distance migrated on the gel, thus allowing the size of the transcripts to be determined. The GPCR-specific probe detected several low abundance transcripts at 12 and 24 hours post-infection. There were two major transcripts of 3.4kb and 4.4kb in length and two lower abundance messages of

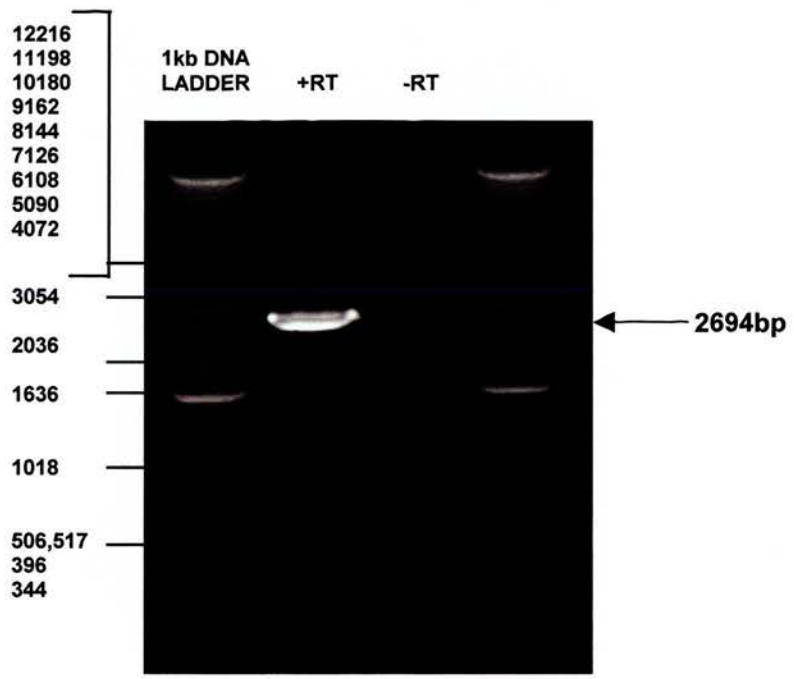
6.6kb and 8.7kb. Since the size of the GPCR coding region is only 1.0kb, it was possible that they represented polycistronic transcripts. The low abundance of the GPCR transcripts prevented their analysis by conventional methods such as S1 nuclease mapping. Therefore the identity of other potential genes encoded on these transcripts was further investigated using RT-PCR as described below.

3.2.3. Co-Transcription of the GPCR with v-Bcl-2

Previous Northern analysis suggested that the polycistronic transcripts detected by the GPCR probe might also encode the MHV-68 homologue of Bcl-2, M11 (data not photographically reproducible). M11 lies approximately 2kb upstream of the GPCR and is transcribed in the same direction (Virgin *et al.*, 1997). It is also expressed at early and late times during lytic infection (Roy *et al.*, 2000). When taken together, these pieces of information suggested that M11 might be co-transcribed with the GPCR.

BHK-21 cells were infected with MHV-68 (5 pfu/cell) and RNA was harvested at 24 hours post-infection. This time-point was chosen on the basis of the Northern analysis, which showed that the GPCR transcripts were most prevalent at 24 hours post-infection. The RNA was reverse transcribed (2.3.10) using random hexamer primers (primer 15, appendix 1) and amplified with a sense primer corresponding to M11 and an anti-sense primer corresponding to the GPCR (pair 5, appendix 1). The result was a 2.7kb product shown in figure 3.2.3. No product was seen when the same reaction was performed in the absence of reverse transcriptase, which confirmed that the DNase treatment had been effective and the 2.7kb band was not derived from contaminating DNA. The PCR product was inserted into the pKS(-) cloning vector and sequenced, revealing that the 2694bp cDNA sequence was identical to the MHV-68 genomic sequence. This would suggest that the transcript was not spliced. However, since the reverse transcription reaction was performed using random hexamer primers as opposed to gene-specific primers, it is not possible to conclude that this transcript originated from the v-Bcl-2 and GPCR coding strand and does not represent a transcript arising from the opposite strand.

Figure 3.2.3. Co-transcription of the GPCR and v-Bcl-2



RT-PCR was performed on cells infected with MHV-68. Using an upper primer specific for v-Bcl-2 and a lower primer corresponding to the GPCR, a 2.7kb PCR product was amplified using *Pfu* polymerase. The PCR products (10μl) were electrophoresed through a 0.8% agarose TAE gel. A negative control that contained no reverse transcriptase (-RT) showed that the product was not amplified from contaminating genomic DNA.

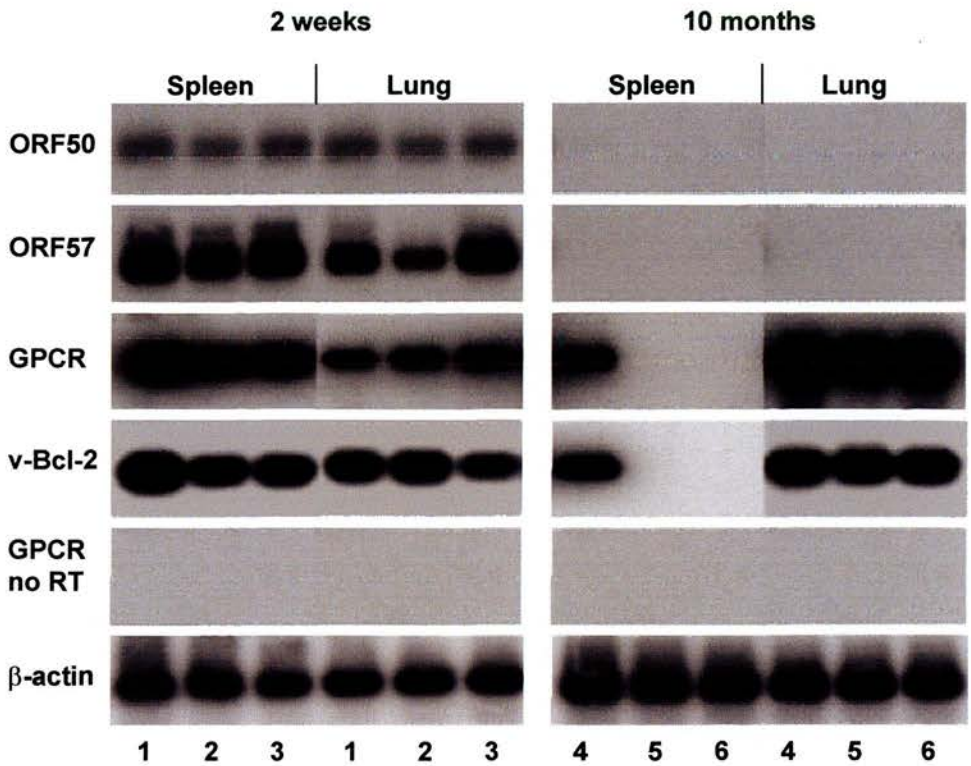
3.2.4. Expression of the GPCR *in vivo*

Expression of the GPCR had previously been detected in the peritoneal cells of mice that were persistently infected with MHV-68, thus suggesting a role for the GPCR in latent infection (Virgin *et al.*, 1999). However, the route of infection utilised in this study was that of intraperitoneal injection, which does not produce an acute infection in the lung. Since it has been demonstrated that the lung is a major site of persistence (Stewart *et al.*, 1998), it seemed logical to confirm this finding in mice that had been infected via the more natural intranasal route. This experiment was performed in conjunction with Dr Douglas Roy and Dr James Stewart (Laboratory for Clinical and Molecular Virology, University of Edinburgh).

Six BALB/c mice were infected intranasally with MHV-68 and viral gene expression in the lung and spleen was analysed using RT-PCR. Tissues were harvested from three mice at 14 days post-infection (p.i.) when the acute infection is resolving in the lung, persistence is being established and latently infected cells are present. The remaining tissues were harvested at 10 months p.i., by which point the virus has established a stable latent infection in the host (Sunil-Chandra *et al.*, 1992a; Sunil-Chandra *et al.*, 1992b).

The RNA was reverse transcribed using random hexamer primers (2.3.10) and amplified with primers corresponding to the coding region of the GPCR (pair 1, appendix 1). The cDNA samples were also amplified with primers specific for v-Bcl-2 (pair 6, appendix 1), as co-expression of v-Bcl-2 and the GPCR had previously been demonstrated *in vitro*. In addition, the expression of ORF50 and ORF57 was investigated (primers described by Virgin *et al.*, 1999). These genes encode transcriptional transactivators that are only transcribed during the virus lytic cycle (Liu *et al.*, 2000; Mackett *et al.*, 1997; Wu *et al.*, 2000). The sensitivity of the PCR reactions was checked by limiting dilution of cloned fragment templates and found to be equivalent and equal to one copy in all cases. No product was seen when the same reaction was performed in the absence of reverse transcriptase, which confirmed that the positive signals did not originate from contaminating DNA. As a control for the integrity of the RNA and efficiency of the cDNA synthesis reaction,

Figure 3.2.4. Expression of the GPCR *in vivo*



In vivo **transcription of the GPCR and v-Bcl-2. Total RNA was extracted from the spleens and lungs of BALB/c mice at 2 weeks and 10 months post infection with MHV-68. RT-PCR was then performed using sets of primers corresponding to viral genes and the cellular gene, β-actin, as indicated at the left. To control for the presence of carry-over genomic DNA, parallel RT-PCR reactions were carried out without reverse transcriptase. The PCR products were electrophoresed through a 1% TAE gel and analysed by Southern blotting. The individual mice used are indicated at the foot of the diagram.**

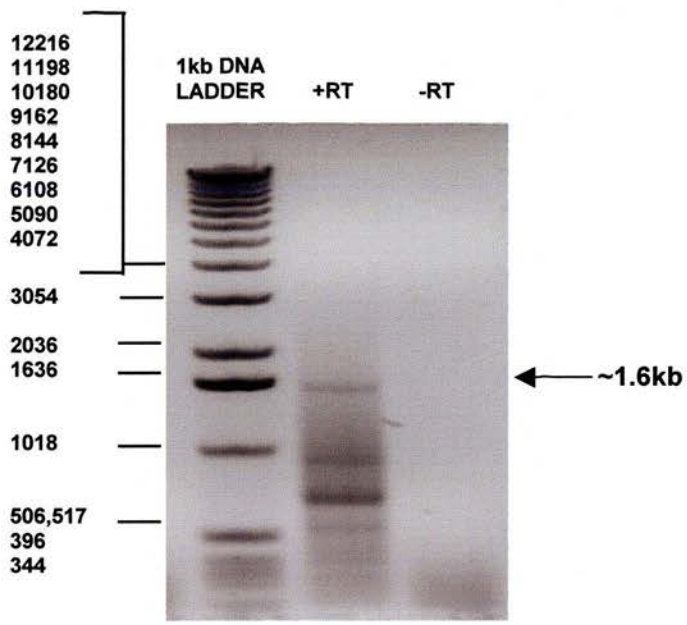
all cDNA samples were amplified with primers specific for the cellular gene, β-actin (pair 19, appendix 1). The PCR products were analysed by Southern blotting using [α^{32}P]dCTP-labelled DNA probes corresponding to each gene product (2.2.20). As shown in figure 3.2.4, transcripts corresponding to the region of the genome encoding ORF50 and ORF57 were detected in both the lung and the spleen at 14 days p.i. in all three mice. However, at 10 months p.i., expression was un-detectable, indicating that productive viral gene expression was still occurring at 2 weeks p.i. but had been completely resolved by 10 months p.i. Expression of transcripts corresponding to the region of the genome encoding the GPCR and v-Bcl-2 were expressed at 14 days p.i. in both the lung and the spleen of all three mice tested, consistent with prior Northern analysis showing transcription of these genes during lytic infection. These transcripts were still detectable at 10 months p.i. in all three lung samples and one spleen sample.

Unfortunately, this experiment does not conclusively prove that the GPCR and v-Bcl-2 are co-expressed during virus latency. Since the reverse transcription was carried out with random hexamers rather than gene-specific primers and the PCR products were detected with double-stranded DNA probes, there is a strong possibility that the transcripts may have arisen from genes on the opposite strand of the virus genome. Although the coding regions of the GPCR and v-Bcl-2 do not overlap with the adjacent genes, ORF73 and ORF75, it is likely that due to the complex nature of transcription in this region, their transcripts may well overlap. Nevertheless, it is quite possible that these transcripts do correspond to the GPCR, v-Bcl-2, ORF50 and ORF57. This being the case, the expression of the GPCR and v-Bcl-2 in the absence of lytic cycle genes such as ORF50 and ORF57 indicates that these transcripts might have originated from latently infected cells. However, further experiments would be required to confirm this finding.

3.2.5. 3'RACE

Having determined that the GPCR and v-Bcl-2 were encoded on the same bicistronic transcript, a 3'RACE technique (2.3.11) was used to investigate the sequence downstream of the GPCR gene and identify the polyadenylation signal. Total RNA

Figure 3.2.5. 3'RACE PCR Products



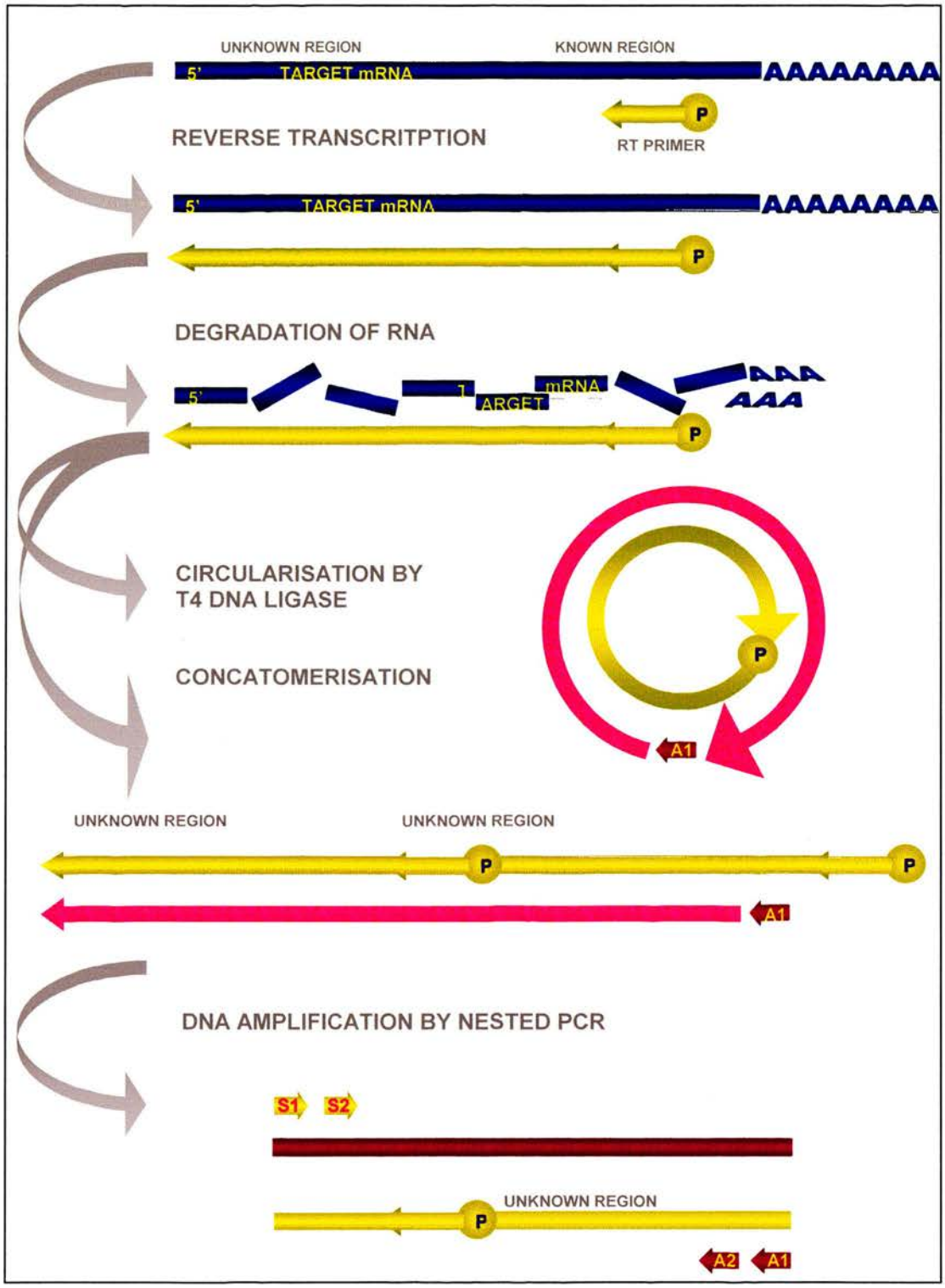
3'RACE was performed on RNA isolated from cells 24 hours post-infection with MHV-68. Reverse transcription was carried out using an oligo(dT) primer. The cDNA was amplified with *Taq* polymerase using the same oligo(dT) primer and a primer complementary to the GPCR coding region. The PCR products (10μl) were electrophoresed through a 0.8% agarose TAE gel. An RT-PCR negative control (-RT) that contained no reverse transcriptase confirmed that the PCR products did not originate from contaminant genomic DNA.

was harvested from BHK-21 cells infected with MHV-68 (5pfu/cell) at 24 hours post-infection. RT-PCR was carried out using an oligo(dT) primer (16, appendix 1), which resulted in selective transcription of polyadenylated RNA. The cDNA was amplified using the same oligo(dT) primer and a primer corresponding to the GPCR coding region (primers 1S and 16, appendix 1). The products of the PCR reaction are shown in figure 3.2.5. The 1.6kb band corresponds to a potential polyadenylation site approximately 600bp downstream of the end of the GPCR coding region at 106622. Southern analysis of the 3'RACE products revealed that all the bands were specific for the GPCR. However, there were no intervening potential polyadenylation sites downstream of the GPCR translation start-site. This suggested that at the low annealing temperature required for this PCR reaction, the oligo(dT) primer hybridised non-specifically with several of the AT-rich regions present in the GPCR coding region.

3.2.6. 5'RACE

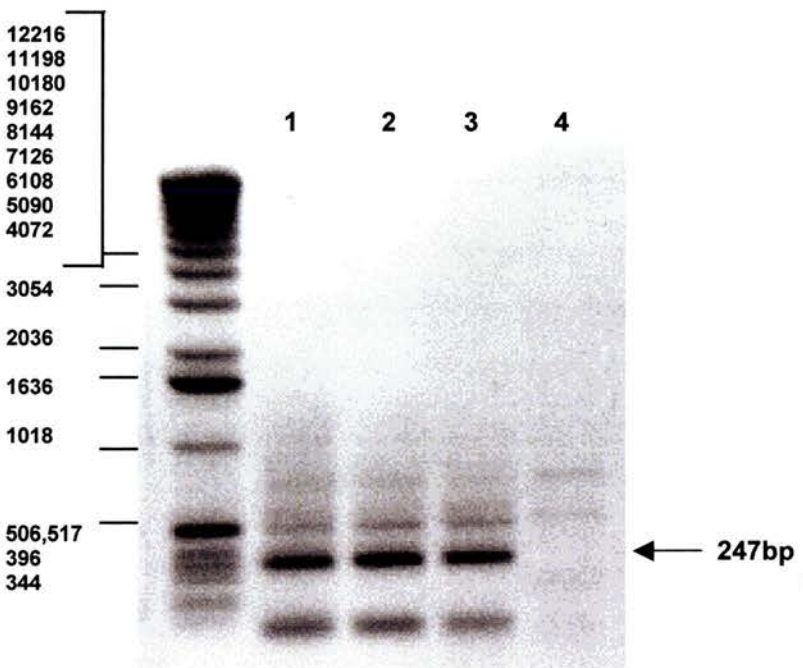
An attempt was made to identify the GPCR transcriptional start-site using a 5'RACE method (2.3.11). A diagrammatic representation of this technique is given in figure 3.2.6A. The target RNA was total RNA harvested from BHK-21 cells infected with MHV-68 at 24 hours post-infection. The RNA was reverse transcribed using a primer (14, appendix 1) that corresponds to the known GPCR coding region. The resulting cDNA extended into the unknown upstream region of the transcript. Once the RNA was degraded using RNaseH, the cDNA underwent a ligation reaction. The presence of the phosphate group on the 5'end of the RT primer facilitated circularisation or concatomerisation of the cDNA molecules. The unknown region, now flanked by regions of known DNA sequence, was amplified using the first round A1 and S1 primers (pair 12, appendix 1). A nested PCR reaction was carried out using the A2 and S2 primers (pair 13, appendix 1). The products of the second round PCR are shown in figure 3.2.6B. The major 247bp band was extracted from the gel and cloned into the vector, pKS (-). Sequencing of the DNA fragment (2.2.24) revealed that it corresponded to the GPCR coding region and did not encode any upstream sequence.

Figure 3.2.6A Diagram of 5'RACE



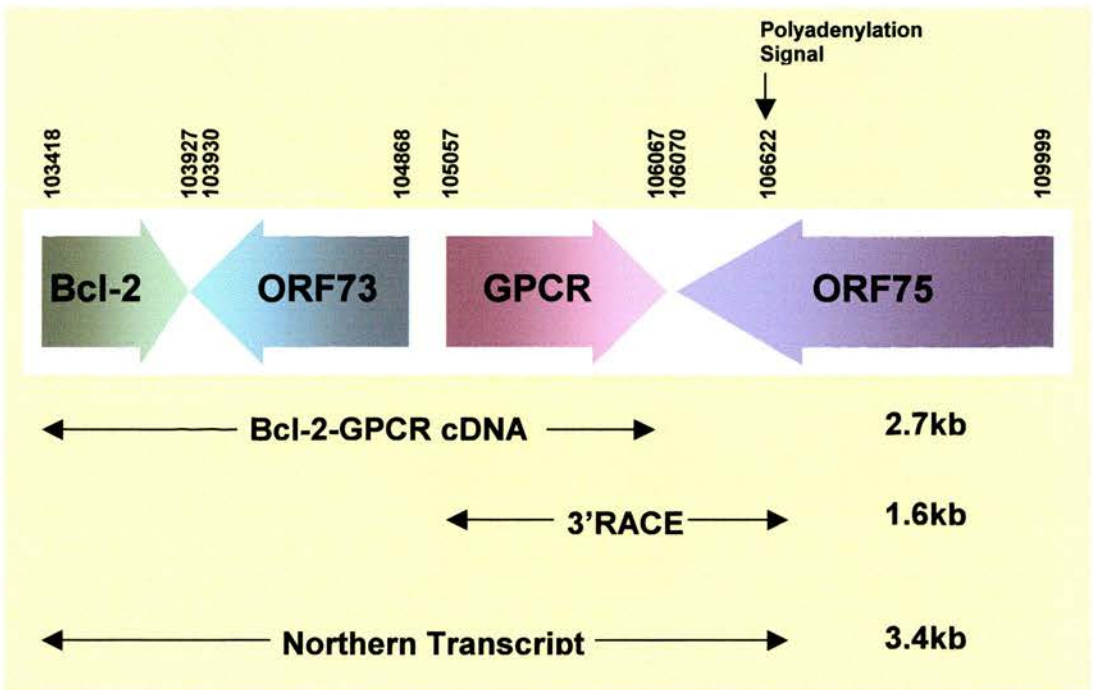
The start site of the bicistronic transcript was investigated using 5'RACE. As shown, this method involves using RT-PCR to extend into the unknown upstream sequence of the transcript. By circularisation or concatomerisation of the cDNA, the unknown sequence is placed between two regions of known sequence thus allowing the 5' region to be amplified.

Figure 3.2.6B 5'RACE PCR Products



5'RACE was carried out using RNA isolated from cells infected with MHV-68. The cDNA was ligated and amplified using *Taq* polymerase. The products of the second round PCR reaction (10μl/lane) were electrophoresed through a 0.8% agarose TAE gel. The DNA template was the product of the first round PCR used neat (lane 1), 1:10 (lane 2) or 1:100 (lane 3). Lane 4 shows a PCR negative control that did not contain DNA.

Figure 3.2.7. Transcriptional Map of the GPCR



This diagram summarises the Northern, RT-PCR and 3'RACE data. Together they suggest that the GPCR and v-Bcl-2 are transcribed bicistronically on a transcript represented by the 3.4kb band on the Northern blot.

3.2.7. Summary of the GPCR Transcription Pattern

Northern analysis revealed that the GPCR was expressed at early and late time-points during MHV-68 lytic infection *in vitro* on rare polycistronic transcripts. The evidence suggested that the GPCR was co-transcribed with the MHV-68 homologue of Bcl-2. Taken together, the RT-PCR and 3'RACE data suggest that the 3.4kb band detected by Northern blotting represents a bicistronic transcript encoding v-Bcl-2 and the GPCR. The results are summarised in figure 3.2.7.

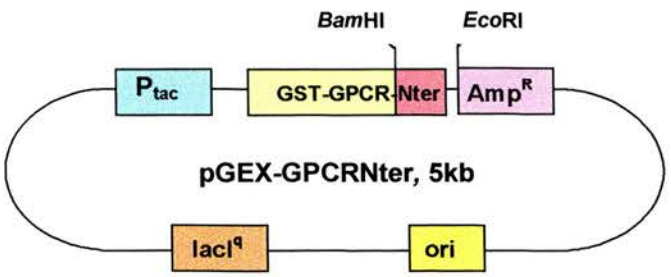
3.3. Expression of the GPCR Protein

3.3.1. Production of a GPCR Fusion Protein

As a first step in the investigation of the GPCR protein, it was decided to raise an antibody. In order to produce an antibody to the GPCR, it was necessary to generate a source of antigen that could be used to raise an immune response *in vivo*. The GPCR was therefore expressed as a GST fusion protein using the pGEX bacterial expression system (Amersham Pharmacia). A 93bp region of the GPCR corresponding to the predicted extracellular NH-terminus was amplified using *Taq* polymerase and primer pair 2 (appendix 1). The DNA fragment was cloned into the pGEX-2T vector, in frame with the *Schistosoma japonicum* glutathione-S-transferase gene under the control of the *tac* promoter (pGEX-GPCRNter, figure 3.3.1A). It was reasoned that the hydrophobic transmembrane domains of the protein were less likely to be immunogenic and were therefore not included in the fusion protein.

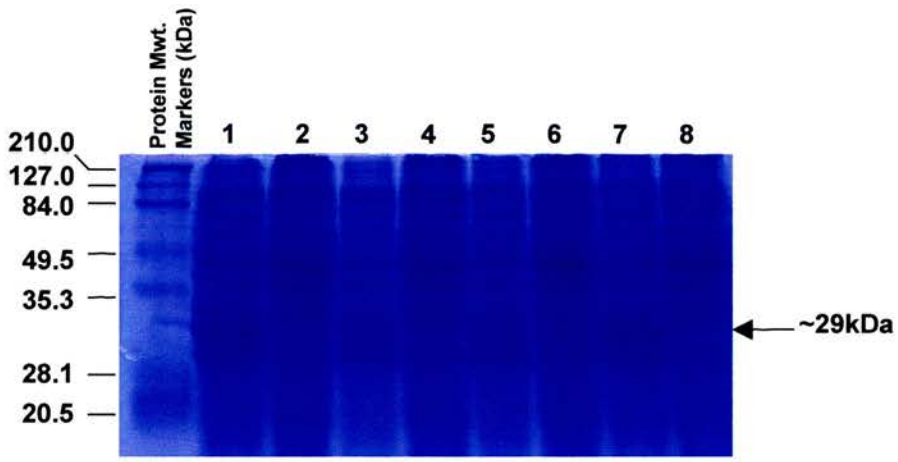
Four pGEX-GPCRNter clones were tested for their ability to express the fusion protein in XL-1 blue bacteria (2.5.1). Expression of the fusion protein was induced by addition of IPTG during exponential growth phase of the culture. Half of each bacterial culture was induced with IPTG while the other half was retained as an un-induced negative control. The cultures were incubated at 37°C for three hours before being pelleted and resuspended in SDS-PAGE sample buffer. The crude bacterial lysates were electrophoresed on an SDS-PAGE gel (2.5.7) as shown in figure 3.3.1B. All four bacterial clones that were induced with IPTG produced a band of approximately 29kDa that was not present in the un-induced negative controls.

Figure 3.3.1A The pGEX-2T Bacterial Expression Construct



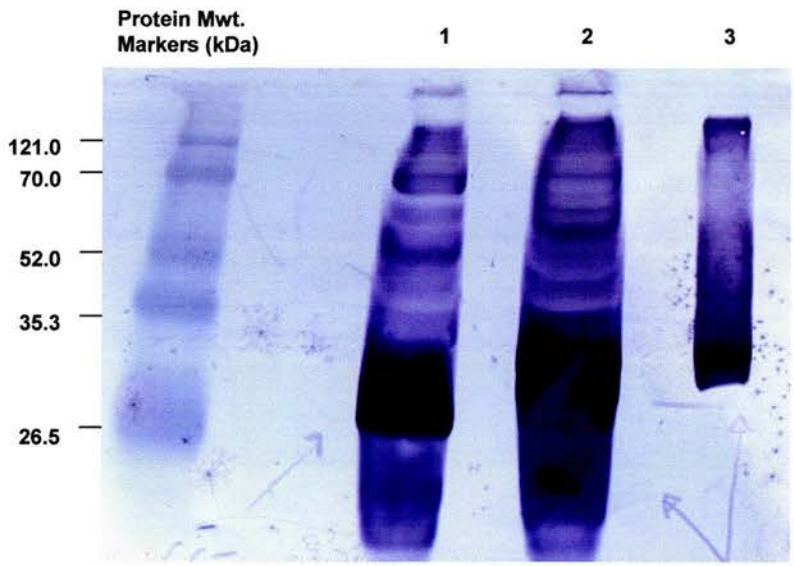
A 93bp DNA region that was predicted to correspond to the extracellular N-terminus of the GPCR was amplified (pair 2, appendix 1) and cloned into the pGEX-2T expression vector in frame with the *S.japonicum* glutathione-S-transferase gene to produce a GST gene fusion. This vector expressed the fusion protein from the inducible prokaryotic *tac* promoter, which is activated upon addition of IPTG. The vector also encodes the *lac* repressor gene (*lacI*^{q}) and an ampicillin resistance gene for selection in bacteria.

Figure 3.3.1B Expression of the Fusion Protein



Four separate GEX-GPCRNter clones were tested for expression of the fusion protein. Crude lysates were electrophoresed on a 15% SDS-PAGE gel. Lanes 1, 3, 5 and 7 contain clones 1-4 that were induced with IPTG, while lanes 2, 4, 6 and 8 contain the un-induced negative controls.

Figure 3.3.1C Western Analysis of the Fusion Protein



Western analysis using an anti-GST antibody. Lane 1: crude lysate of bacteria carrying the empty pGEX-2T vector and induced with IPTG to express GST. Lane 2: crude lysate of bacterial clone no.1, induced to express the fusion protein. Lane 3: fusion protein that was purified using GS beads. The blot was incubated with a rabbit anti-GST antibody, washed and incubated with swine anti-rabbit-AP antibody. A colorimetric reaction was produced by addition of NBT/BCIP.

Since the size of the fusion protein was predicted to be 3kDa larger than GST (26kDa), it was probable that this band represented the GST-GPCRNter fusion protein. The expression level of the fusion protein appeared to be equivalent in each clone.

To confirm that this band represented the fusion protein, a Western blot was generated that was incubated with an anti-GST antibody. The IPTG-induced crude bacterial lysate corresponding to clone no.1 was electrophoresed on an SDS-PAGE gel. A crude lysate of bacteria expressing GST from an empty pGEX-2T vector was electrophoresed on the same gel. The protein was transferred to a Western blot as described in section 2.5.8 and incubated with a rabbit anti-GST antibody (Stewart, unpublished). This was followed with a secondary swine anti-rabbit antibody conjugated to alkaline phosphatase. Finally, the blot was incubated with NBT/BCIP to produce a colorimetric reaction (figure 3.3.1C). The anti-GST antibody reacted strongly with the pGEX-2T negative control to produce a band of approximately26kDa that corresponds to GST. The antibody also bound to a band in the region of 29kDa that was likely to be the GST-GPCRNter fusion protein.

An identical Western blot was produced that was incubated with a rabbit polyclonal anti-MHV-68 antibody (Sunil-Chandra *et al.*, 1992a). The same secondary antibody was used and the blot was incubated with NBT/BCIP as before. The anti-MHV-68 antibody, however, did not recognise the GPCR region of the fusion protein (data not shown).

3.3.2. Purification of the Fusion Protein

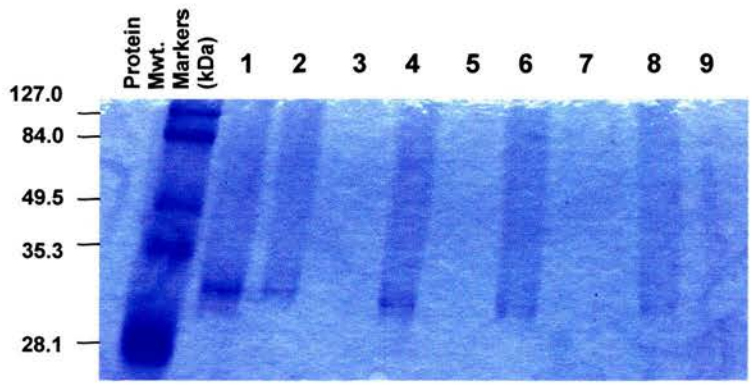
The fusion protein was isolated using an affinity purification system that involved glutathione-sepharose (GS) beads (2.5.2). To test whether the fusion protein was soluble in aqueous solution and bound to the GS beads, clone no.1 was cultured and induced with IPTG. The bacteria were lysed by sonication and the soluble fraction was incubated with the GS beads. The beads were washed, resuspended in SDS-PAGE sample buffer and loaded onto an SDS-PAGE gel. The protein was transferred to a nylon membrane by Western blotting and incubated with anti-GST

antibody as described above (figure 3.3.1C). The anti-GST antibody recognised the purified fusion protein thus showing that it was present in the soluble fraction and bound to the GS beads.

The next step in the purification process was to elute the fusion protein from the GS beads using a glutathione solution to compete with the glutathione sepharose for the GST binding site. To test this, bound GS beads were mixed with 5mM glutathione as recommended by the manufacturer (Amersham Pharmacia). The beads were pelleted by centrifugation and the supernatant removed. Both fractions were mixed with SDS-PAGE sample buffer and electrophoresed on an SDS-PAGE gel. No protein was visible in the eluate: the fusion protein remained attached to the GS beads. This experiment was repeated using clone no.2 and the same result was obtained. The concentration of glutathione was increased from 5mM to 10mM and 15mM but this did not effect elution (figure 3.3.2).

It was surmised that since the protein could be visualised on an SDS-PAGE gel, it must be removed from the beads by boiling in SDS-PAGE sample buffer. However, it was with reluctance that the stringency of the elution was increased as raising the temperature or salt concentration might denature the protein and therefore abrogate any chance of the antibody recognising the native GPCR protein. It was also deemed inappropriate to use SDS or other detergents that could not be removed by dialysis and would therefore render the antigen unsuitable for application *in vivo*. Nevertheless, attempts were made to elute the fusion protein using a range of concentrations of glutathione (5-100mM), sodium chloride (0.1-3.0M) or urea (3.0-5.0M). The protein-bound GS beads were also heated to 100°C in a solution of 15mM glutathione but no protein was ever visualised in the eluate. It was observed that some fusion protein was still present in the insoluble fraction after sonication of the bacteria. This suggested that the fusion protein was only partially soluble which might prevent elution into an aqueous solution. Therefore, it was decided to use the fusion protein as an antigen while still bound to the GS beads.

Figure 3.3.2. Failure to Elute the Fusion Protein



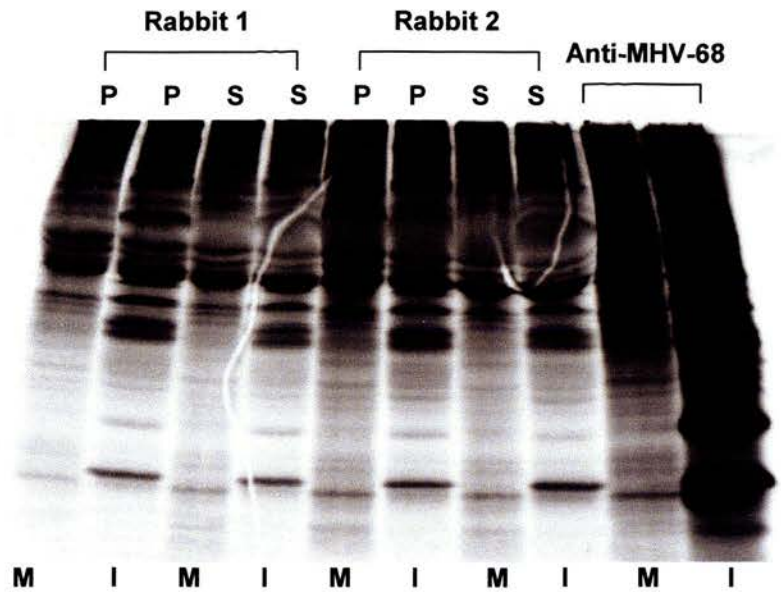
Testing elution of the fusion protein from GS beads. Aliquots of GS beads bound to the GST-GPCRNter fusion protein were mixed with elution buffer. The beads were pelleted and resuspended in SDS-PAGE sample buffer while the eluate was mixed 1:1 (v/v) with sample buffer. The eluted beads and eluates were electrophoresed through a 12% SDS-PAGE gel. Lane 1: un-eluted beads. Lane 2: beads treated with 10mM glutathione and corresponding eluate (lane 3). Lane 4: beads with 15mM glutathione and eluate (lane 5). Lane 6: beads with 15mM glutathione + 1M NaCl and eluate (lane 7). Lane 8: beads eluted with 15mM glutathione + 3M NaCl (lane 9).

3.3.3. Analysis of Antisera

The fusion protein was purified on a large scale and two rabbits were immunised with a mixture containing the bound GS beads and adjuvant (2.5.3). After three weeks the sera were tested against cells infected with MHV-68 (24 hours p.i.) by Western blotting. When compared with the pre-immunisation sera samples, the antisera displayed no specific reactivity with the virus-infected cell sample (not shown). The rabbits received three booster injections at 3-week intervals and the antisera was tested by Western blotting between each injection. Although the antisera generated by both rabbits recognised the GST-GPCRNter fusion protein, they failed to recognise the native GPCR protein in MHV-68 infected cells or in a stable 3T3 clone expressing the GPCR (GPCR1, section 3.4.3). The potentially low level of GPCR protein expression in the infected and stably transfected cells may have been beyond the sensitivity limit of the Western analysis. Therefore, the more sensitive radio-immunoprecipitation assay was used.

BHK-21 cells were infected with MHV-68 and radiolabelled with ^{35}S-methionine at 12 hours p.i. when, according to Northern analysis, the GPCR was expressed (2.5.9). Un-infected cells were also radiolabelled as a negative control. The cells were lysed and the lysates incubated with antisera and pre-immune sera from each rabbit in addition to a positive control anti-MHV-68 antibody. In order to isolate the antigen-antibody complexes, the lysates were incubated with protein A sepharose, which binds to the Fc receptors of immunoglobulin molecules. The protein A sepharose beads were washed, pelleted and electrophoresed on an SDS-PAGE gel. The gel was dried and exposed to autoradiographic film (figure 3.3.3). The samples incubated with the positive control anti-MHV-68 antibody confirmed that the BHK-21 cells had efficiently incorporated the radiolabelled methionine. The difference between the infected and un-infected samples (lanes 9 and 10) showed that there was also efficient labelling of MHV-68 proteins. However, in neither of the rabbit samples was there any specific difference between the antisera and the pre-immune sera that was also specific to the MHV-68 infected cells. This indicated either the GPCRNter portion of the fusion protein was not sufficiently immunogenic or that any antibody specific for the fusion protein failed to recognise the native GPCR protein.

Figure 3.3.3. Radio-Immunoprecipitation Assay to Test Antisera



BHK-21 cells were infected with MHV-68 and radiolabelled with [^{35}S]-methionine and incubated for 4 hours (37°C). The cells were lysed in RIPA buffer and the lysates pre-cleared by incubating with sepharose. Lysates were incubated with rabbit test sera or anti-MHV-68 antibody and precipitated by mixing with protein A sepharose. The sepharose beads were washed, resuspended in SDS-PAGE sample buffer and electrophoresed through a 12% SDS-PAGE gel. P: Pre-immune sera, S: Test sera, I: Infected, M: Mock Infected.

Figure 3.3.4A Location of Peptides in the GPCR Amino Acid Sequence

Two peptides were designed that corresponded to the GPCR amino acid sequence. The peptide sequences were selected on the basis that they represented hydrophilic, extracellular regions of the protein, which were more likely to be immunogenic. The hydrophilic and extracellular domains of the GPCR protein were predicted using a sequence analysis programme, "TMpred" (Hofman, 1993). GPCR2 encoded the residues highlighted in grey within the dotted line, thus joining two hydrophilic regions with a small hydrophobic region.

3.3.4. Generation of Antibody using Peptides

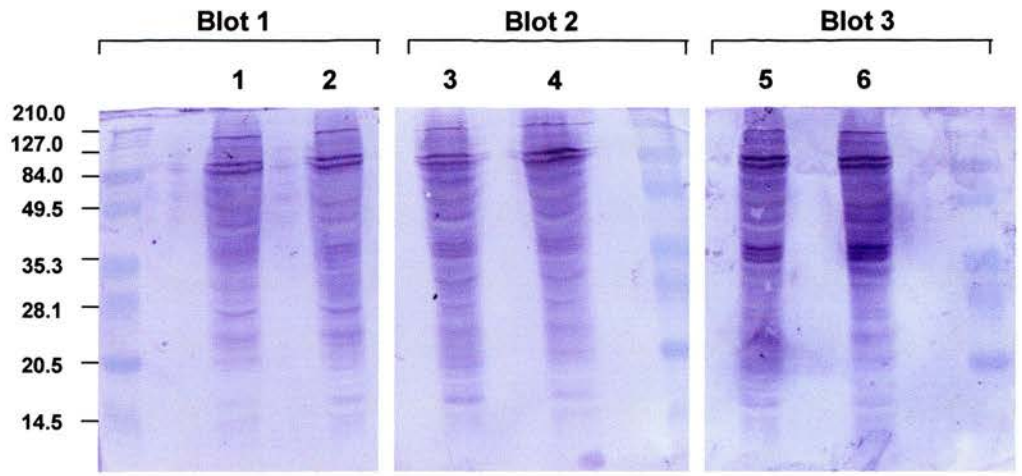
The inability of the rabbit sera to recognise the GPCR protein prompted a new immunisation strategy involving the use of peptides as antigen. Two peptides were synthesised that corresponded to hydrophilic, extracellular regions of the GPCR amino acid sequence (figure 3.3.4A) and GPCR1 was coupled to bovine serum albumin as described in section 2.5.6. However, prior to immunisation with the peptides, two sheep were vaccinated with DNA encoding the GPCR. Each sheep received four intra-muscular injections of 25μg pVR1255-GPCRHA3 (figure 3.3.5A) in a volume of 250μl. One sheep was also immunised with DNA encoding the cytokine, GMCSF (4x 25μg pEGFP-GMCSF), in an attempt to enhance the immune response to the GPCR. The sheep received a booster injection of DNA after three weeks. Nine weeks after the initial DNA vaccination, the sheep were injected intramuscularly with 25μg peptide (12.5μg GPCR1 + 12.5μg GPCR2) in a volume of 250μl at four sites. The antisera were tested against 293 cells that were transiently transfected with pVR1255-GPCRHA3 by Western analysis but no specific bands were apparent (figure 3.3.4B).

3.3.5. Epitope-Tagging of the GPCR

In the absence of an antibody to the GPCR, the sub-cellular localisation of the protein was investigated using an "epitope-tagging" strategy. A version of the GPCR was produced that encoded an epitope derived from the influenza virus haemagglutinin protein at the carboxy terminus. It was anticipated that expression of this gene in transfected cells could then be detected using an anti-haemagglutinin antibody. The GPCR coding region was amplified using an antisense primer incorporating a 27bp DNA sequence that corresponds to an epitope of the influenza virus haemagglutinin protein (pair 3, appendix 1). The sequence of nine amino acids was inserted immediately upstream of the GPCR STOP codon so that it would be translated in-frame with the receptor.

The PCR product was inserted into the mammalian expression vector pBABE/*puro* (appendix 2) under the control of a retroviral LTR, to give pBabe-GPCRHA (figure 3.3.5A). The construct was transfected into NIH3T3 cells via electroporation (2.4.3)

Figure 3.3.4B Western Analysis of Sheep Antisera



Western analysis to test the reactivity of sheep antisera to the GPCR protein. 293 cells were transiently transfected with pVR1255-GPCRHA3 and harvested at 24 hours post-transfection. Cells were resuspended in SDS-PAGE sample buffer and electrophoresed through a 12% SDS-PAGE gel. A blot of the gel was incubated with the sheep antiserum (1:1000) followed by biotinylated donkey anti-sheep antibody and streptavidin-AP. A colorimetric reaction was produced by addition of NBT/BCIP. Lanes 1, 3 and 5: 293 cells expressing the GPCR. Lanes 2, 4 and 6: negative control mock-transfected cells. Blots 1 and 2 were incubated with anti-serum from the sheep immunised with GPCR-derived peptides. As a negative control, blot 3 was incubated with serum taken from a non-immunised sheep.

and stable transfectants were selected by treating with 3μg/ml puromycin as described in section 2.4.5.

Both transient and stably transfected cells were stained using a standard immunofluorescence technique described in section 2.5.10. No positive control for the anti-haemagglutinin antibody was available at this time. Un-transfected 3T3 cells were used as a negative control. The slides were incubated with rat anti-haemagglutinin antibody (1:1000, Roche) for 1 hour at room temperature then washed before adding the secondary antibody: FITC-labelled goat anti-rat polyclonal antibody (1:250, Pharmingen). No specific FITC staining was detected in cells transfected with the GPCR therefore a "bridging" method was employed to increase the sensitivity of the assay. Following incubation with the primary antibody, the cells were washed and incubated with a secondary biotinylated anti-rat antibody (Dako). The cells were then exposed to streptavidin conjugated with alkaline phosphatase (streptavidin-AP, Roche) and a colorimetric reaction was produced by addition of NBT/BCIP. Again, no specific staining was detected using a light microscope. It was concluded that either the level of GPCR protein expression was below the limits of detection or that a single haemagglutinin epitope provided insufficient binding sites for the antibody.

To improve the detectable level of GPCR expression, a new expression construct was generated containing three haemagglutinin epitopes. The GPCR was amplified using primers (pair 4, appendix 1) that incorporated three identical consecutive haemagglutinin epitopes at the 3' end of the GPCR. The DNA was inserted into the mammalian expression vector, pVR1255^{luc-} (appendix 2), under the control of the CMV promoter (pVR1255-GPCRHA3, figure 3.3.5A). This vector, which was designed for the high-level expression of inserted genes, was obtained under license from Vical Inc. (Hartikka *et al.*, 1996).

Prior anecdotal evidence had indicated that the transfection efficiency of 293 cells was superior to that of 3T3 cells. The triple-tagged GPCR construct was transfected into 293 cells via electroporation and the cells were harvested at 24 hours post-

Figure 3.3.5A Haemagglutinin-tagged Expression Constructs

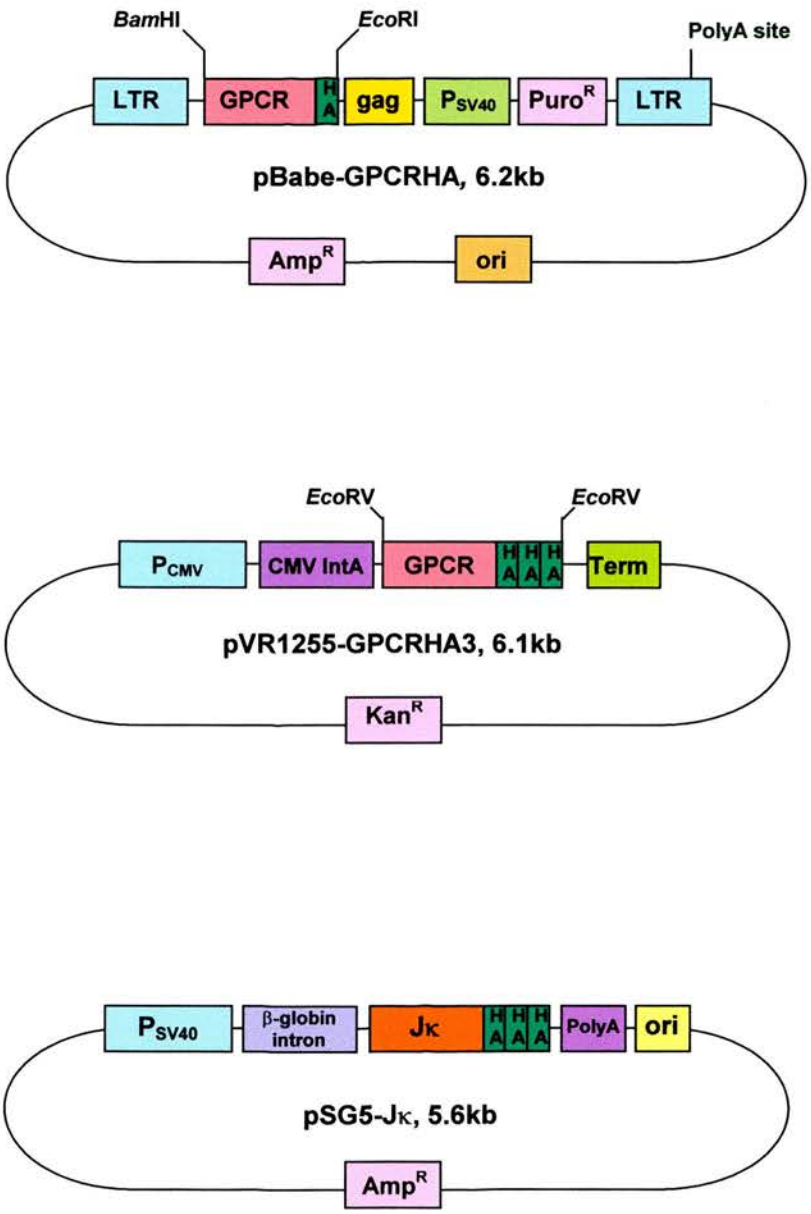


Figure 3.3.5A. To generate pBabe-GPCR, the GPCR was amplified using a primer that incorporated a 27bp sequence encoding a single epitope derived from the influenza virus haemagglutinin protein (HA) onto the 3' end of the gene. This HA-tagged GPCR was inserted into the multiple cloning site of the pBabe/*puro* retroviral expression vector under the control of the Moloney murine leukaemia virus long terminal repeats (LTR). This vector encodes an ampicillin resistance gene (Amp^R) for selection in bacteria and a puromycin resistance gene ($Puro^R$) for selection in mammalian cells. A polyadenylation site (PolyA) is present within the downstream LTR. A non-functional MuLV gag gene is included as a packaging signal for the production of recombinant virus in a complementary helper cell line. However, in these experiments, the pBabe/*puro* vector was used solely as an expression plasmid.

For construction of pVR1255-GPCRHA3, the GPCR was amplified using a primer that incorporated three consecutive HA epitopes at the 3' end of the gene. The triple HA-tagged GPCR was inserted by blunt-end cloning into the *Eco*RV site of the $pVR1255^{luc-}$ expression vector. This vector contains the CMV IE enhancer/promoter upstream of the CMV intron A, cloning sites and a rabbit β-globin polyadenylation site. There is also a kanamycin resistance gene for selection in bacteria but no selective marker for use in mammalian cells.

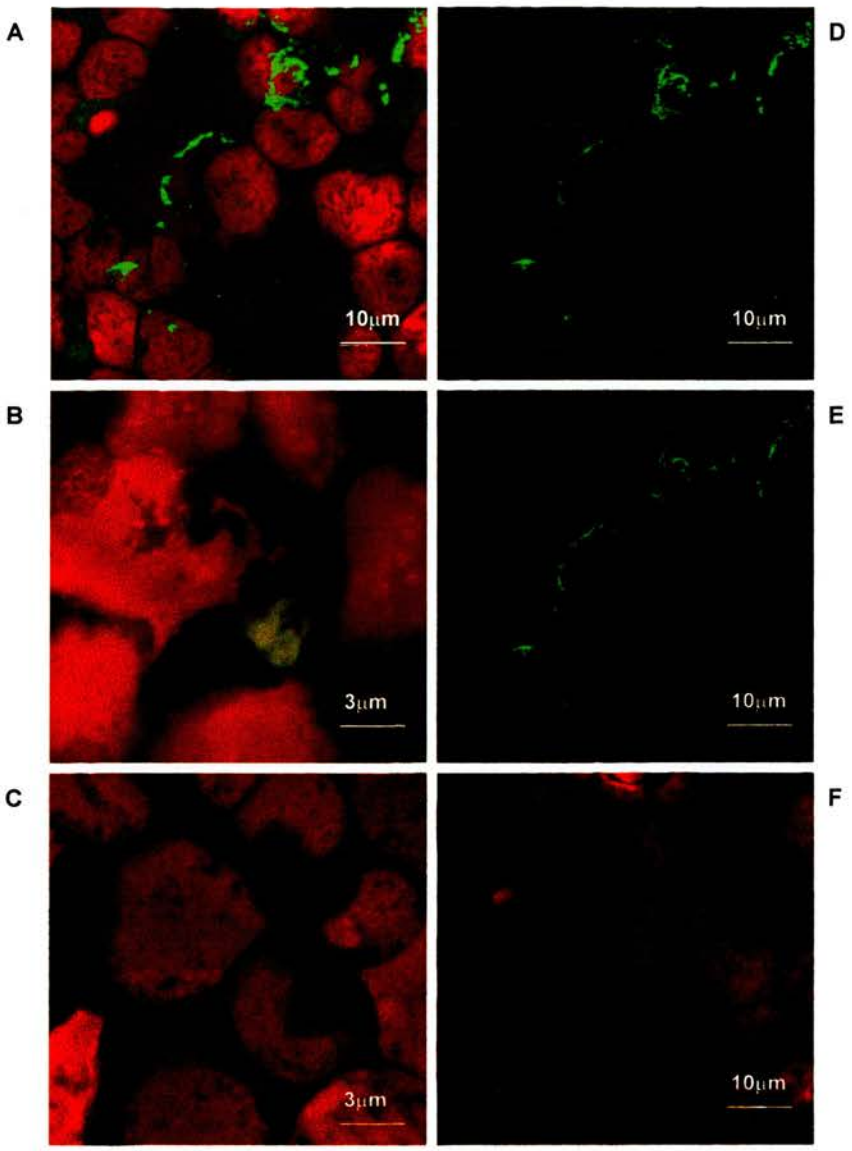
A triple HA-tagged Jκ contained in the pSG5 expression vector (pSG5-Jκ) was used as a positive control for haemagglutinin expression. pSG5 expresses inserted genes from the SV40 early promoter and includes intron II of the rabbit β-globin gene, a polyadenylation signal and ampicillin resistance gene.

transfection. The cells were fixed and stained with the rat anti-haemagglutinin and FITC-labelled anti-rat antibodies as before. Mock-transfected 293 cells were used as a negative control. As a positive control for the anti-haemagglutinin antibody, cells were transfected with a triple HA-tagged version of the cellular signalling protein, Jκ, inserted into the pSG5 (Stratagene) expression vector obtained from Dr Clare Sample (Zhao *et al.*, 1996).

A small percentage of cells transfected with pVR1255-GPCRHA3 construct showed specific FITC staining. The experiment was repeated and the cell nuclei were counterstained red with propidium iodide. As shown in figure 3.3.5B, the anti-haemagglutinin antibody detected patches of GPCRHA3 quite separate from the intra-nuclear propidium iodide staining. This staining pattern was consistent with expression of the GPCR at the plasma membrane. Transfection of 293 cells with the pSG5-Jκ construct resulted in characteristic nuclear staining with the anti-haemagglutinin antibody whereas the negative control mock-transfected cells failed to show positive staining.

The low percentage of cells exhibiting immunofluorescence did not reflect the efficiency of transfection. A positive control plasmid expressing GFP in a parallel transfection demonstrated a transfection efficiency of 50-60%. This implies that the level of GPCR expression in the majority of cells is below detectable limits in this assay. As discussed below, this protein appears to be toxic to bacteria and mammalian cells. This suggests that cells that have not taken up the plasmid and cells expressing only very low levels of the protein may have a selective advantage following transfection. Therefore in this context, the original pBabe-GPCRHA construct may have been almost impossible to detect. Expression of the GPCRHA gene was driven by a retroviral LTR, which is a weaker promoter than the CMV immediate early promoter used to drive expression of the GPCR in pVR1255-GPCRHA3. In addition, pBabe-GPCRHA encoded only one binding site for the anti-haemagglutinin antibody compared with three sites in pVR1255-GPCRHA3.

Figure 3.3.5B Expression of the GPCR in 293 Cells



Composite confocal image of 293 cells transiently transfected with pVR1255-GPCRHA3 (A). The cells were harvested at 24 hours post-transfection, fixed and stained with an anti-haemagglutinin antibody (rat anti-haemagglutinin antibody, clone 3F10, Roche) followed by a FITC-labelled goat anti-rat antibody. The cell nuclei were counterstained red using propidium iodide. Panels D, E and F show three of the single slice images of which the composite image in panel A is composed. Panel B shows the characteristic nuclear staining of the HA-tagged positive control, pSG5-Jκ. Mock transfected negative control cells are shown in panel C.

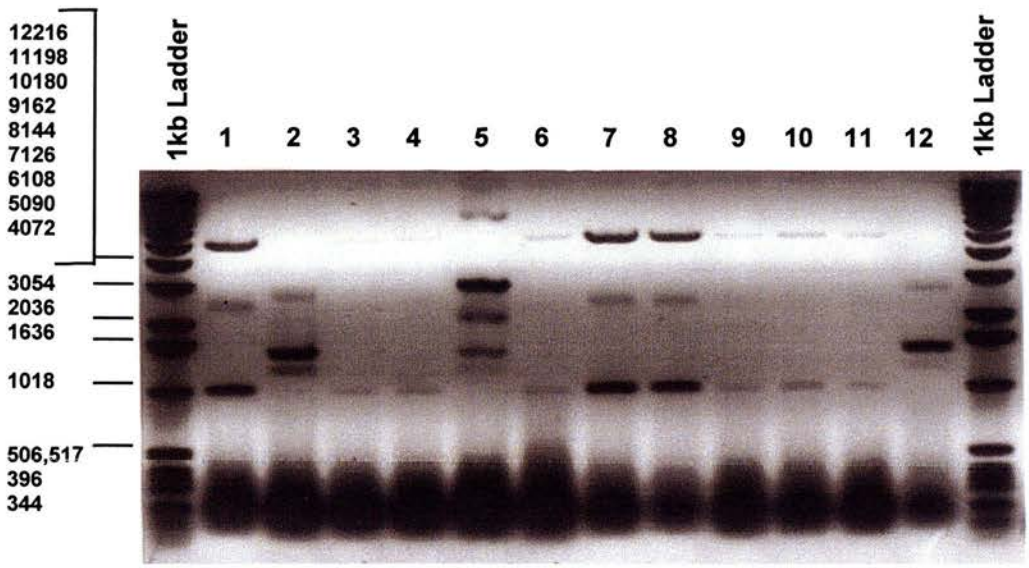
3.3.6. Toxicity of the GPCR

The insertion of the GPCR into expression vectors and subsequent expression of the protein in mammalian cells has revealed the toxicity of this gene in both bacteria and eukaryotic cells. Cloning of the GPCR into the pBabe/*puro* vector was a prolonged process and the bacterial clone obtained did not grow rapidly in culture. In addition, the gene was subject to rearrangement in this vector. When a bacterial glycerol stock of the pBabe-GPCR clone was streaked onto an LB-agar plate containing ampicillin, large rapidly growing colonies were produced alongside the characteristically small colonies. A mixture of colonies was cultured and DNA was extracted and digested with appropriate restriction enzymes to excise the GPCR insert. As shown in figure 3.3.6, only 50% of the clones (nos.3, 4, 6, 9, 10 and 11) were fully digested even with addition of excess enzyme and an extended incubation period. This suggested that a rearrangement of the DNA had occurred resulting in loss of the restriction sites. Therefore, prior to large-scale preparation of pBabe-GPCR, the clone was streaked on an LB-agar plate and the DNA in individual colonies tested by restriction digest. The pBabe-GPCR clone used subsequently in transformation experiments (section 3.5) was sub-cloned into the pKS (-) vector and sequenced to ensure that it was free of mutations (2.2.24). Transfection of mammalian cells (3T3 and 293 cells) with expression vectors encoding the GPCR (pBabe-GPCR and pVR1255-GPCRHA3) resulted in fewer viable cells at 24 hours post-electroporation compared with control vectors (i.e. empty pBabe/*puro*). The number of cells expressing the GPCR (<1%), as detected by immunofluorescence, was also well below the transfection efficiency of a positive control vector expressing GFP (50-60%). This suggests that expression of the GPCR is toxic to mammalian cells when expressed at high levels.

3.3.7. Binding of IL-8 to the GPCR

The closest homologue of the MHV-68 GPCR is the mammalian chemokine receptor, CXCR2. CXCR2 binds the CXC chemokines IL-8, GROα and NAP-2 with high affinity. The KSHV and HVS GPCR homologues have both demonstrated chemokine-binding activity *in vitro* (Ahuja & Murphy, 1993; Arvanitakis *et al.*, 1997). The HVS GPCR exhibits a similar cytokine profile to CXCR2 while the

Figure 3.3.6. Toxicity of the GPCR in Bacteria



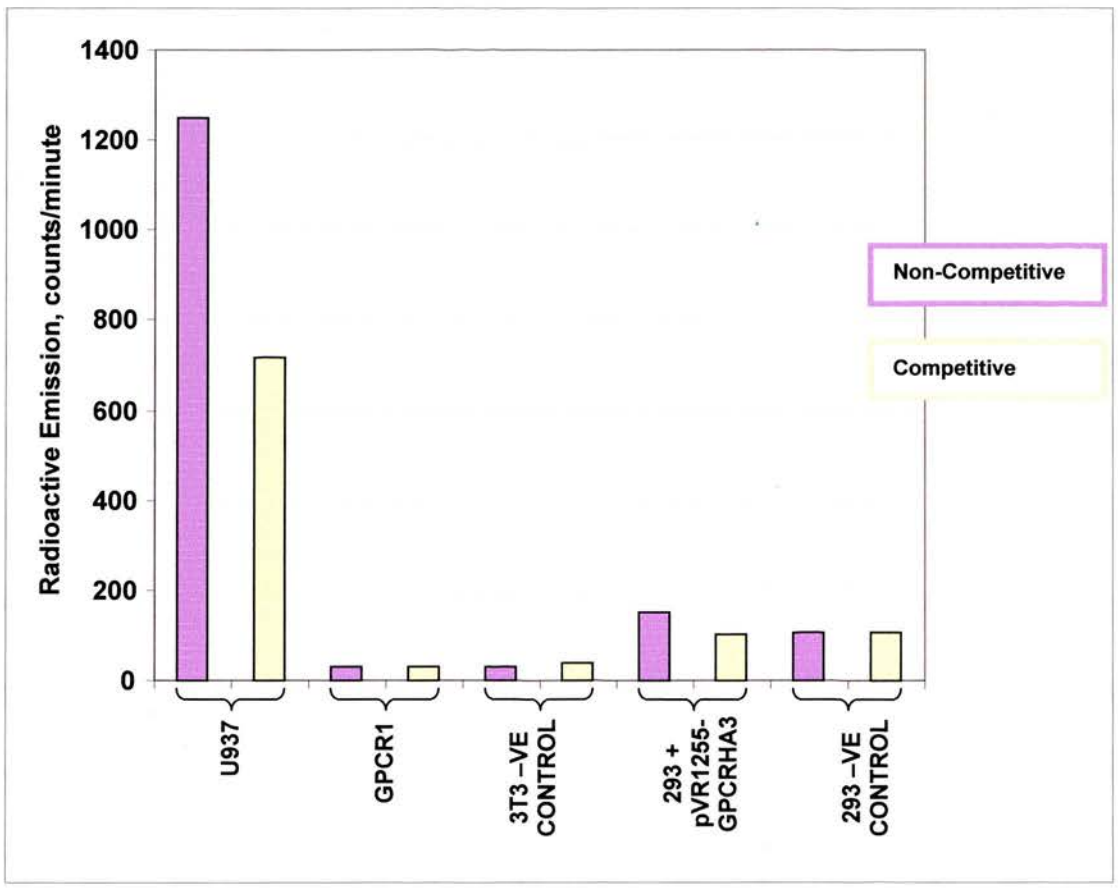
A bacterial glycerol stock of the pBabe-GPCR (see figure 3.3.5A) clone was streaked on an LB agar plate containing ampicillin. Twelve colonies were picked and grown. DNA was extracted (2.2.14) and digested with restriction enzymes (*Bam*HI and *Eco*RI) to excise the GPCR insert.

KSHV GPCR appears to be a more promiscuous receptor with the capacity to bind both CXC and CC chemokines. Nevertheless, both receptors bind IL-8 with high affinity, which made it reasonable to speculate that the MHV-68 GPCR might also bind IL-8.

The potential interaction between the MHV-68 GPCR and IL-8 was investigated using a competitive ligand-binding assay (2.4.16). Stably transfected 3T3 cells expressing the GPCR (GPCR1, section 3.4.3) were incubated with radiolabelled human recombinant [^{125}I]IL-8. Un-transfected 3T3 cells were used as a negative control and all samples were tested in triplicate. The lymphoma cell line, U937, was used as a positive control. U937 cells, which possess monocyte-like characteristics, have been shown to bind IL-8 and are often used as a positive control in this type of assay (Milne *et al.*, 2000). All cells were extensively washed to remove unbound ligand then solubilised with sodium hydroxide. Radioactive emission was estimated using a liquid scintillation counter. To control for non-specific binding, a parallel assay was performed in which cells were incubated with [^{125}I]IL-8 in the presence of a thousand fold excess of unlabelled IL-8. Competitive inhibition of radiolabelled cytokine with the "cold" unlabelled cytokine is indicative of specific binding.

The GPCR1 cells consistently failed to show specific binding to [^{125}I]IL-8 (figure 3.3.7). This suggested that either the MHV-68 GPCR does not interact with IL-8 or that there was insufficient expression of the GPCR to demonstrate binding. The assay was also performed using 293 cells that were transiently transfected with the pVR1255-GPCRHA3 expression construct. However, there was no significant difference between cells transfected with GPCRHA3 DNA and mock-transfected negative controls. Incubation of U937 cells with [^{125}I]IL-8 produced a strong radioactive signal that was reduced in the presence of unlabelled IL-8, thus confirming that the parameters of the assay were appropriate for detection of chemokine binding. As discussed in the preceding sections, expression of the GPCR in mammalian cells appears to be toxic. It is likely that expression of the GPCR was at barely detectable levels in both the transiently and stably transfected cells. Therefore, it is not clear using this assay whether the GPCR binds mammalian IL-8.

Figure 3.3.7. Competitive Binding Assay



Competitive binding of [^{125}I]IL-8 to cells expressing the GPCR. Cells were incubated with radiolabelled IL-8 alone or in the presence of excess unlabelled IL-8. The radioactive emission generated by the bound [^{125}I]IL-8 was measured using a liquid scintillation counter (Wallac) in counts/min. The results are expressed as the mean of the three determinations ± standard deviation from the mean. A two-sample students' T-test revealed a significant difference between the competitive and non-competitive positive control U937 samples with a confidence level of 95% where a value of P = 0.05 is significant (P = 0.019). There was no significant difference between cells expressing the GPCR and negative controls.

3.4. Transforming Activity of the GPCR

3.4.1. Generation of Stable Cell Clones

The description of a transforming activity associated with the KSHV GPCR raised the possibility that the MHV-68 GPCR might also have the capacity to transform cells (Arvanitakis *et al.*, 1997; Bais *et al.*, 1998). However, despite the predicted structural similarity of these proteins, the level of sequence identity between the KSHV and MHV-68 receptors is not high (25% amino acid identity). It was therefore necessary to assess whether these viral GPCRs shared functional similarity.

The potential transforming activity of the GPCR was investigated using tissue culture techniques, which addressed two different criteria of cellular transformation: loss of contact inhibition and anchorage independence. Since conventional assays for transformation have been based on fibroblast cells, transformation has been defined by a set of differences in the growth of fibroblastic cells in culture (Tooze, 1973). The growth of normal fibroblast cells is regulated by contact from neighbouring cells and it has been shown that cell contact plays a role in inhibiting cell division (Dulbecco, 1970). Primary fibroblast cells and the cells of most fibroblastic cell lines also require attachment to a solid surface in order to divide, a phenomenon termed anchorage dependence of multiplication (Stoker *et al.*, 1968). In transformed cells, these growth control signals are de-regulated resulting in loss of contact inhibition and anchorage-independent growth. The focus formation assay was used to demonstrate loss of contact inhibition in cells expressing the GPCR and anchorage-independence was assessed in the soft agar assay.

In order to express the GPCR in cells, it was essential to insert the gene into an expression vector. Therefore, the GPCR gene was amplified (pair 1, appendix 1) and inserted into the retroviral expression vector, pBabe/*puro* (appendix 2) under the control of the Moloney murine leukaemia virus (MoMuLV) long terminal repeats. This vector was chosen on the basis that it would express the GPCR at a relatively physiological level in transfected cells. The transformation construct is shown in figure 3.4.1. Two positive controls for transformation were used: the EBV latent

membrane protein (LMP-1) gene and the SV40 virus early genome region, which includes the large T antigen. LMP-1 is expressed in EBV-transformed lymphoblastoid cell lines and is essential for EBV-induced B cell transformation (Kaye *et al.*, 1993). LMP-1 has also been shown to transform rodent fibroblast cells *in vitro* (Wang *et al.*, 1985). A region of DNA encoding the LMP-1 gene was excised as a *Bam*HI fragment from a construct available in the laboratory (pAT153, (Stewart *et al.*, 1989)) and sub-cloned into the pBabe/*puro* plasmid.

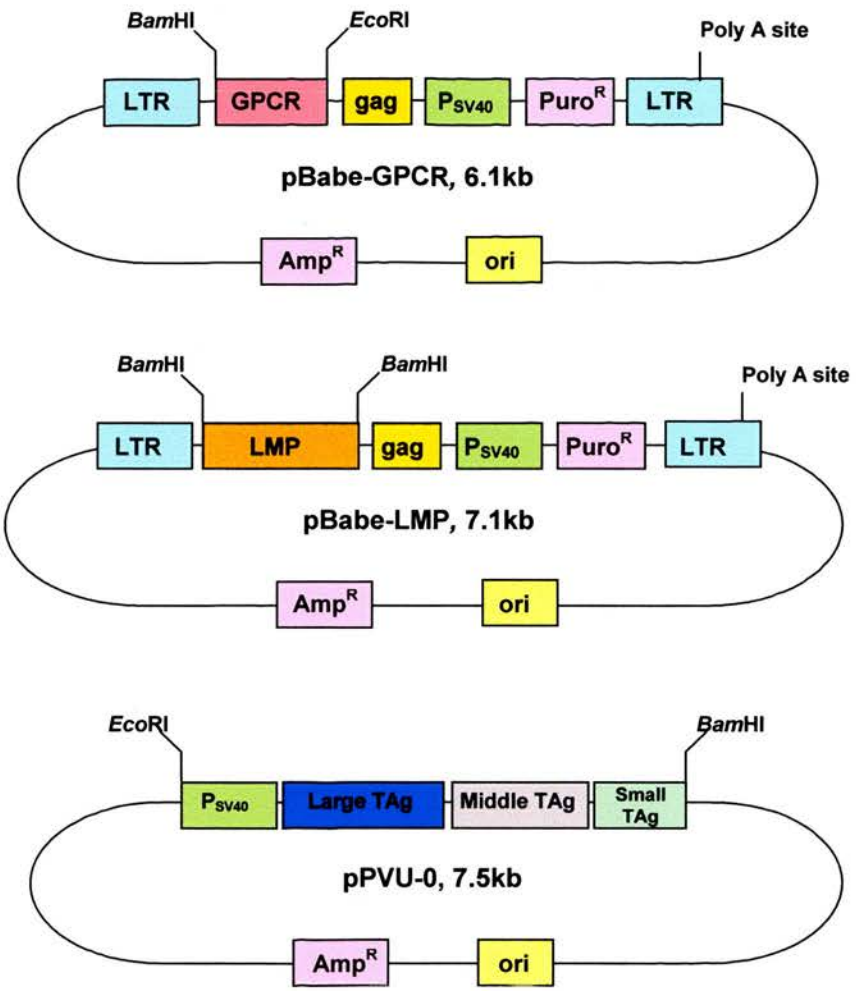
The SV40 early region encodes the small, middle and large T antigen (TAg). During productive SV40 infection, large TAg regulates the level of virus gene expression and stimulates virus replication. The large T antigen has been shown to be necessary and sufficient for transformation of rodent cells *in vitro* (Tooze, 1973). The pBR328 construct containing the SV40 early region (pPVU-0) was obtained from Paul Brickell, Middlesex Hospital Medical School, London (Kalderon *et al.*, 1982). All three transformation constructs are depicted in figure 3.4.1.

A clone of NIH3T3 cells (Bojan *et al.*, 1983) that had been selected for their lack of growth in soft agar and formation of foci was obtained from Dr Simon Stacey, Paterson Institute for Cancer Research, Manchester. Given that cell-cell contact promotes cellular transformation (Todaro, 1963), cells were passaged when only 80% confluent to minimise the risk of spontaneous transformation. Cells ($2x10^6$) were transfected with 20μg of each expression construct (pBabe-GPCR, pBabe-LMP, pPVU-0 or empty pBabe/*puro* plasmid) as described in section 2.4.3. The medium was replaced every 2-3 days and foci of puromycin resistant cells were observed after 7 days. Resistant cellular foci were cloned using a modified method described by Puck, 1956 (section 2.4.5). 3T3 clones were propagated in medium containing 1μg/ml puromycin to prevent reversion to a puromycin-sensitive phenotype.

3.4.2. Focus Formation Assay

The puromycin-selected stable 3T3 clones and cells that were transiently transfected with the pPVU-0 plasmid were assessed for a transformed phenotype using the focus

Figure 3.4.1. Constructs used in Transformation Assays



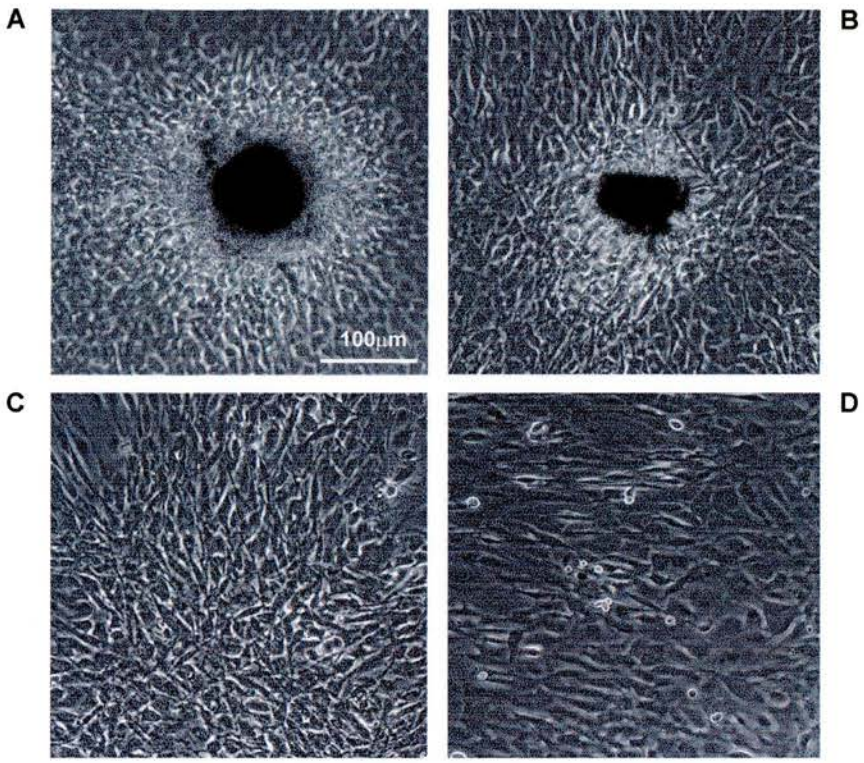
The retroviral-based plasmid vector, pBabe/*puro*, (Morgenstern & Land, 1990) expresses genes inserted in the MCS under the control of the Moloney murine leukaemia virus (MoMuLV) long terminal repeats (LTR). It encodes the ampicillin resistance gene (Amp^R) for selection of the plasmid in bacteria and the puromycin resistance gene ($Puro^R$) that is driven by the SV40 promoter for selection in mammalian cells. A polyadenylation site is present in the downstream LTR. A disabled MuLV gag gene is included as a packaging signal for when the vector is used to generate recombinant virus in a complementary cell line. In these experiments, the pBabe/*puro* vector has been used solely as an expression plasmid. The GPCR and LMP-1 genes were inserted into the plasmid multiple cloning site as shown. The pPVU-0 construct is comprised of a DNA region encoding the SV40 early region inserted into the pBR328 plasmid, which resulted in the loss of the *Pvu*II restriction site (Kalderon *et al.*, 1982). This plasmid contains an ampicillin resistance gene for selection in bacteria.

formation assay (2.4.6). Each clone was maintained as a confluent monolayer in triplicate wells of a 6-well plate in DMEM containing 10% foetal calf serum. The medium was replaced on alternate days for 3-4 weeks until cellular foci were observed using phase-contrast light microscopy (figure 3.4.2). Cells that were stably transfected with the pBabe-GPCR or pBabe-LMP constructs, or transiently transfected with pPVU-0, exhibited signs of transformation. The cell monolayers contained regions of cells that were not contact-inhibited and whose growth had extended over neighbouring cells (figure 3.4.2B). In places, these areas of loss of contact-inhibition had developed into cellular foci (figure 3.4.2A). The 3T3 clones carrying only the empty pBabe/*puro* vector did not display this transformed phenotype (figure 3.4.2C).

3.4.3. Generation of Transformed 3T3 Clones

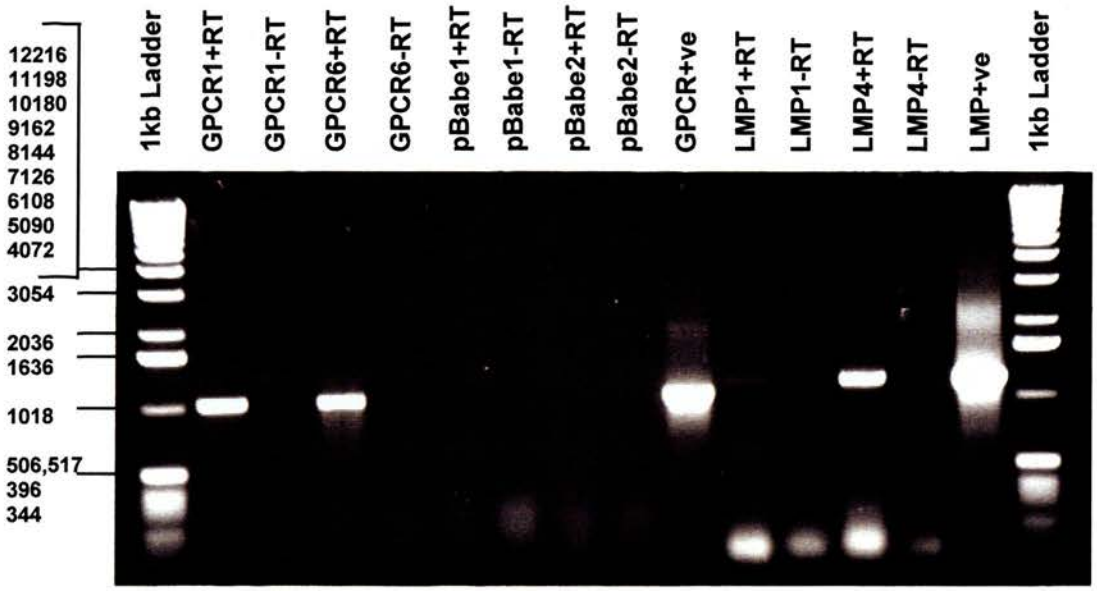
Individual cellular foci produced by four of the puromycin-resistant cell lines in the focus formation assay were cloned as previously described (2.4.5), to generate stably transformed cell lines. The expression of the GPCR or LMP-1 in each of these cell lines (GPCR1, GPCR6, LMP1, LMP4) was confirmed using RT-PCR (figure 3.4.3). Total RNA was harvested from cells using a kit (RNeasy® Mini Kit, QIAGEN, 2.3.3) and reverse transcription was carried out as described in section 2.3.10. The cDNA was amplified with *Taq* polymerase using primers specific for the GPCR (pair 1, appendix 1) or the LMP-1 coding region (pair 18, appendix 1). Two stable puromycin-resistant cell clones carrying only the empty pBabe/*puro* vector tested negatively for expression of the GPCR by RT-PCR. The 3T3 cells transiently transfected with pPVU-0 produced a smaller number of foci due to the low efficiency of transfection. Two cellular foci were cloned and propagated. Punctate nuclear expression of the large T antigen was detected by immunofluorescence (2.5.10) in one cell clone (SV402) using an anti-SV40 large T antigen monoclonal antibody (TCS Biologicals). A small percentage of cells (1-2%) exhibited a low level of positive staining that was not photographically reproducible.

Figure 3.4.2. Focus Formation Assay



The transforming activity of the GPCR was investigated using the focus formation assay. 3T3 cells were transfected with pBabe-GPCR, pBabe-LMP, pBabe/*puro* or pPVU-0. Stably transfected 3T3 clones expressing the GPCR or LMP-1 were generated by puromycin selection. Cells ($1x10^6$) were seeded in 30mm 6-well plates in DMEM containing 10% FCS. The medium was replaced on alternate days and the cells were maintained in a confluent monolayer for a period of 3-4 weeks. Changes in cellular morphology were observed using phase-contrast light microscopy. Stable 3T3 clones expressing the GPCR or LMP-1, or transiently transfected with pPVU-0, exhibited loss of contact inhibition (C = GPCR1) and formation of cellular foci (A = GPCR1; B = LMP4). 3T3 clones stably transfected with the pBabe/*puro* vector did not display any signs of transformation (D). Images A, B, and C are shown at equivalent magnification with the size bar indicating approximately 100μm.

Figure 3.4.3. GPCR and LMP-1 Expression in Transformed 3T3 Clones



The expression of the GPCR or LMP-1 was confirmed in stably transfected, transformed 3T3 cell clones (GPCR1, GPCR6, LMP1 and LMP4 respectively). Total RNA was harvested from cells ($5x10^6$) and 2μg each sample was reverse transcribed using Superscript II reverse transcriptase (Life Technologies) as described in section 2.3.10. The GPCR coding region was amplified using specific primers (pair 1, appendix 1) from the cDNA obtained from the GPCR1 and GPCR6 cell clones. LMP-1 was amplified using primers corresponding to the coding region (pair 18, appendix 1) from cDNA generated from the LMP1 and LMP4 cell clones. As positive controls, the GPCR was amplified from MHV-68 DNA (100ng) and LMP-1 was amplified from pBabe-LMP DNA (100ng). No PCR products were generated using the negative control (-RT) cDNA samples thus showing that the RT-PCR products were not derived from contaminating DNA. Two stably transfected negative controls (pBabe1 and pBabe2) tested negatively for expression of the GPCR. The PCR products ($^1/_{10}$ reaction volume) were electrophoresed on a 0.8% agarose TAE gel.

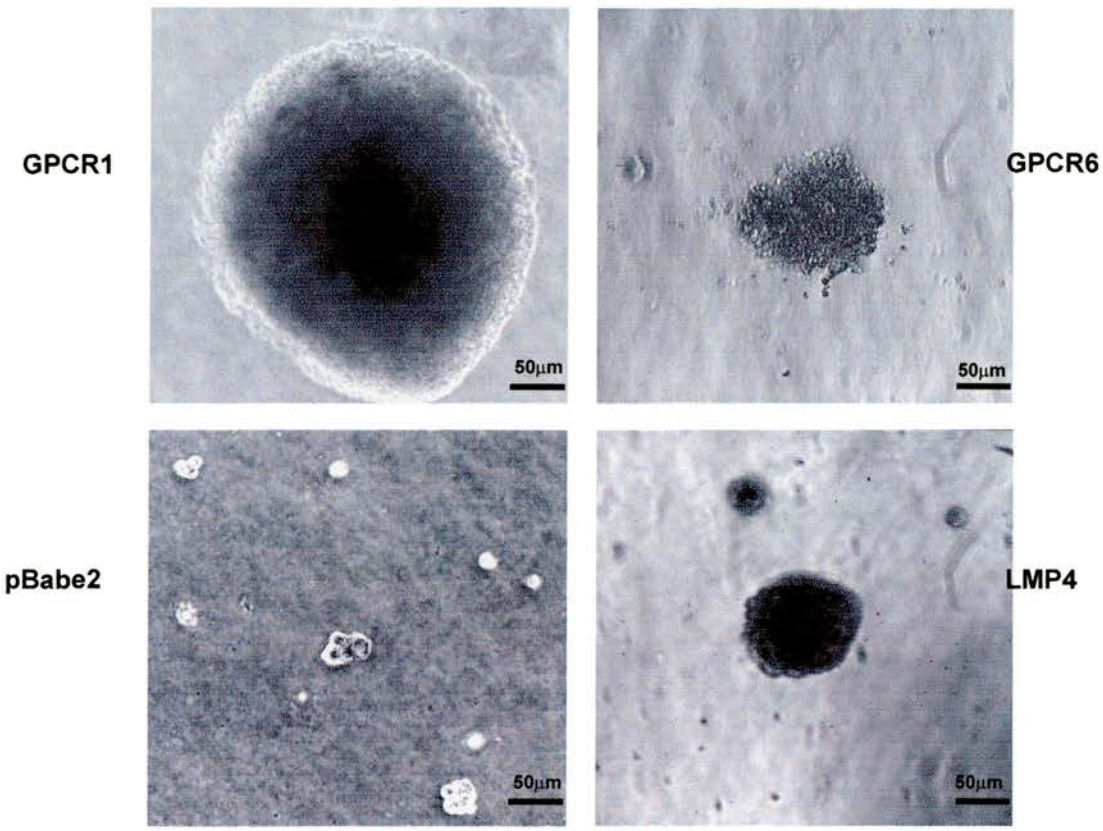
3.4.4 Growth in Soft Agar

The anchorage independent growth of the stably transformed 3T3 clones, GPCR1, GPCR6, LMP1, LMP4 and SV402 was demonstrated by growing the cells in soft agar (Baichwal & Sugden, 1989). Six-well plates were layered with 2ml 0.5% low gelling temperature agarose. Cells ($1x10^4$) were resuspended in 2ml DMEM + 10% FCS containing 0.35% agarose and layered on top of the supporting layer. Two stable cell clones carrying the empty pBabe/*puro* vector (pBabe1 and pBabe2) and un-transfected 3T3 cells were used as negative controls. Each cell clone was cultured in triplicate wells and supplemented with 2-3 drops medium on alternate days. After a period of 2-3 weeks, cellular foci were visualised using phase contrast light microscopy (figure 3.4.4A) and counted with the aid of a calibrated graticule. Since normal 3T3 cells went through one or two rounds of abortive division, only foci of 50μm or greater in diameter were counted. The results, expressed as a mean of three determinations ± standard deviation from the mean, are shown in figure 3.4.4B. The 3T3 clones expressing the GPCR (GPCR1 and GPCR6), LMP-1 (LMP1 and LMP4) and the SV40 early region (SV402) all produced significantly more foci than the negative controls, as measured using the students' two sample t-test (table 3.4.4).

3.4.5 Growth in Low Serum

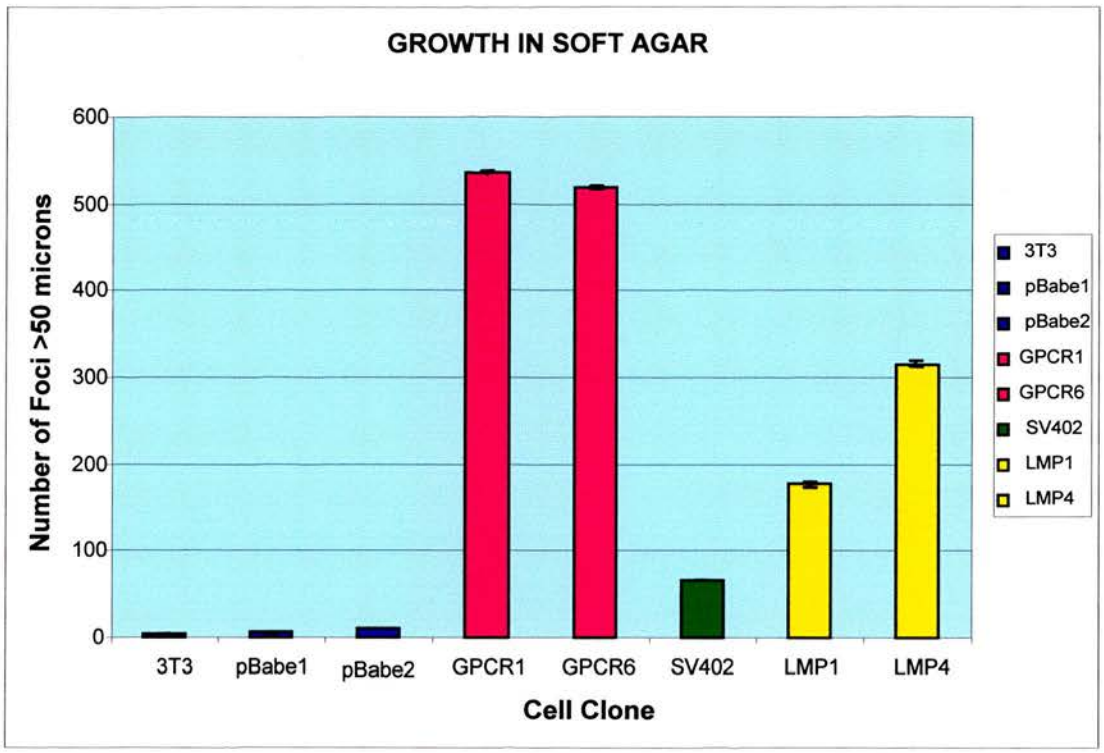
Transformed cells may also acquire the ability to grow and divide in the absence of serum or with a lower serum requirement than normal cells. This is known as serum independent growth. The stable cell clones expressing the GPCR, LMP-1 or SV40 early region were tested for their ability to grow with a reduced serum supplement. Cells were maintained at a range of serum concentrations (0.1%-5%) but the appropriate conditions required for growth of the transformed cells and concomitant death of the negative controls were not found. This may reflect the particular sensitivity of this assay or simply reveal that serum independent growth was not a property of the transformed cell clones assayed here.

Figure 3.4.4A Anchorage Independent Growth



Cell clones (GPCR1, GPCR6, LMP1, LMP4, SV402, pBabe1 and pBabe2) and un-transfected 3T3 cells were grown in soft agar according to a method described by Baichwal & Sugden, 1989. After 3 weeks, cellular foci were visualised in 3T3 clones expressing the GPCR, LMP-1 or SV40 early region e.g. GPCR1, GPCR6 and LMP4 (above). The negative control cells (3T3, pBabe1 and pBabe2) did not produce cellular foci. However, they did proceed through one or two rounds of abortive division e.g. pBabe2. Cells were visualised using phase contrast light microscopy with the aid of a calibrated graticule.

Figure 3.4.4B Growth of Transformed 3T3 Clones in Soft Agar



The transformed 3T3 clones (GPCR1, GPCR6, SV402, LMP1 and LMP4) and negative controls (pBabe1, pBabe2 and un-transfected 3T3 cells) were grown in soft agar. Cells ($1x10^4$) were grown in triplicate 6-well plates containing DMEM supplemented with 10% FCS and 0.35% LGT agarose (Seaplaque®, Flowgen). After three weeks, the number of cellular foci generated by each cell line, with diameter equal to or exceeding 50μm, was counted. The small size of the error bars indicates the minimal amount of variation between the samples.

3.4.6 Tumorigenicity in Nude Mice

Another classical transformation assay involves injection of transformed cells into mice to assess their ability to form tumours. In order to prevent rejection, cells are injected into nude mice, which are athymic and therefore lack B and T lymphocytes (Pantelouris, 1968). As the KSHV GPCR had been shown to be tumorigenic in nude mice (Bais *et al.*, 1998), and the MHV-68 GPCR had demonstrated a transforming activity *in vitro*, the tumourigenicity of stable $GPCR^+$ cells was investigated. Six groups of four six-week old BALB/c *nu/nu* mice were injected subcutaneously in the left flank with $1x10^6$ cells (GPCR1, GPCR6, LMP4, SV402, pBabe2 and un-transfected 3T3 cells) in a volume of 100µl as described in section 2.4.8.

The mice were observed for 3-4 weeks, during which time no tumours developed. The experiment was repeated by injecting $1x10^7$ cells, of the same cell clone as previously received, into the right flank of each mouse. After a period of 1-2 weeks, the mice injected with 3T3 clones expressing the GPCR, LMP-1 or SV40 early region showed some evidence of tumour formation. The negative control mice, which had received either pBabe2 or un-transfected 3T3 cells, did not exhibit any signs of tumorigenesis. However, the tumorigenesis was transient in nature and all the mice experienced regression of their tumours within the following week.

3.5. Recombinant Virus

3.5.1. Design of the Recombinant Virus Construct

One approach taken to investigate the potentially pathogenic role of the GPCR *in vivo* was to generate a virus lacking this gene. Comparison of a GPCR "knock-out" virus with the wild type virus would provide insights into its role during infection. As shown in figure 3.5.1A, a homologous recombination strategy was employed to replace the GPCR with a marker gene, the *Aequorea victoria* green fluorescent protein (GFP). This permitted the selection of recombinant virus plaques expressing GFP under UV illumination. The goal in these experiments was firstly to generate a $GPCR^-$ recombinant virus and secondly to purify it to the standard at which wild type virus was not detectable by PCR. Ultimately, the intention was to infect mice with

Table 3.4.4. Statistically Significant Growth of Transformed Clones

3T3 Clone	P Value
pBabe1	0.5400
pBabe2	0.0013
GPCR1	< 0.0001
GPCR6	< 0.0001
SV402	0.0006
LMP1	< 0.0001
LMP4	< 0.0001

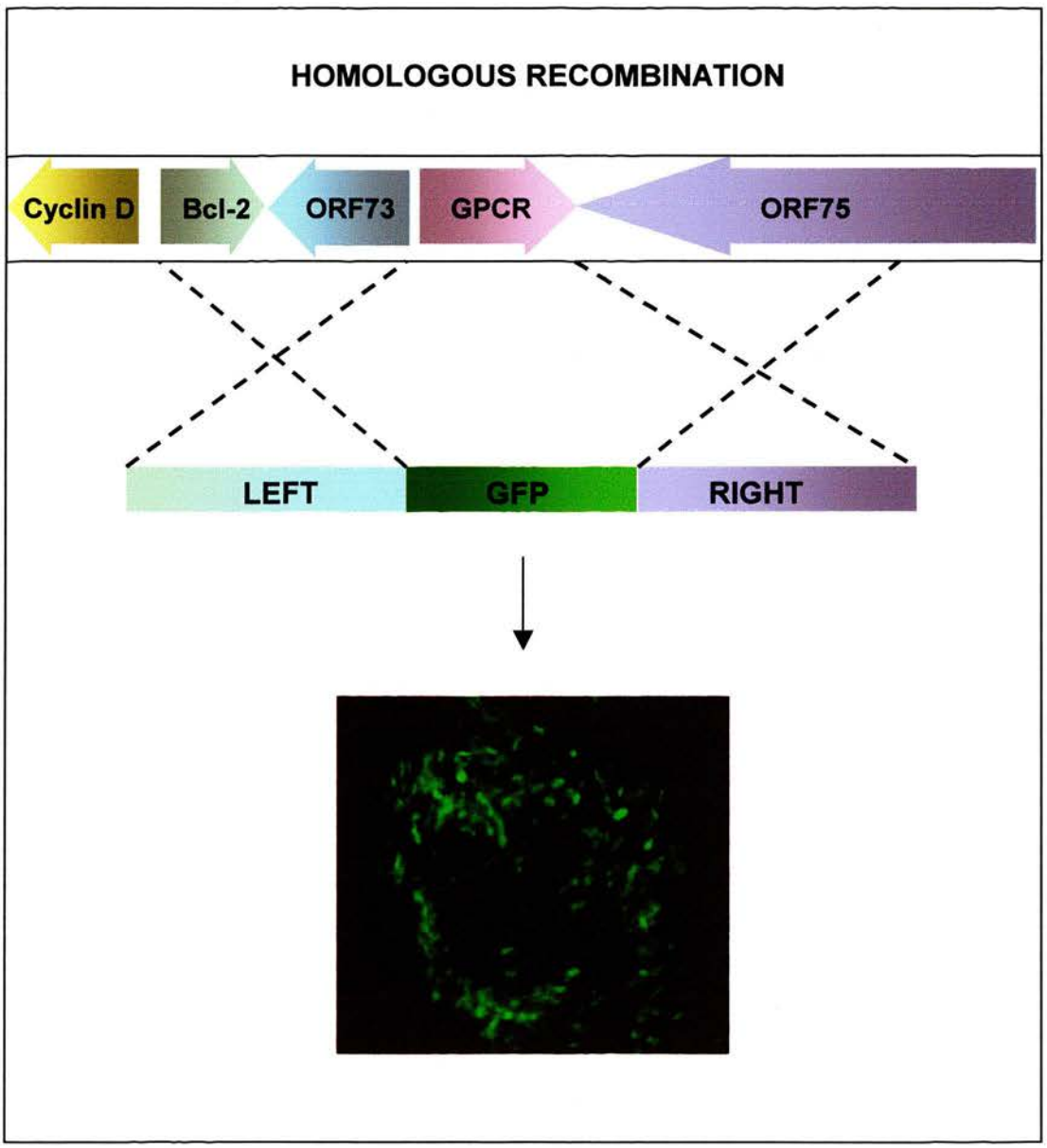
Growth of stably transfected cell clones in soft agar. A two-sample student's T-test was used to compare the number of colonies produced by the 3T3 clones listed above with that of ordinary 3T3 cells. A P value of less than 0.05 indicates a statistically significant difference with a confidence interval of 95%.

pure recombinant virus and compare it with wild type virus infection by titration of infectious virus in the lung and reactivation of latent virus in the spleen.

A DNA construct was designed which would recombine with MHV-68 DNA resulting in deletion of the GPCR (Figure 3.5.1B). In designing the construct, it was necessary to consider whether deletion of the GPCR would lead to disruption of other open reading frames. The genes adjacent to the GPCR, ORF73 and ORF75, do not overlap with the GPCR, therefore it appeared feasible to delete the entire GPCR gene without disrupting adjoining coding regions. There is a distance of approximately 200bp between the start of the GPCR coding region and the ORF73 coding region, which is transcribed in the opposite direction. Therefore, it is possible that the GPCR coding region contains promoter or enhancer elements corresponding to ORF73 or ORF75, which, if deleted could modify their expression. In addition, replacement of the GPCR with a marker gene could alter the chromosomal structure thus affecting expression of other genes.

With these caveats in mind, a recombination construct was produced, comprising two regions of DNA homologous to the sequences flanking the GPCR bisected with the marker gene, GFP. The size of homologous flanking regions chosen for the construct was based on previous experiments with vaccinia virus. Recombinant vaccinia viruses were generated using recombination cassettes with flanking regions of only 500bp. Similarly, it has been possible to produce recombinant HSV-1 viruses using relatively short regions of homology. Preliminary attempts to generate MHV-68 recombinant viruses suggested that 500bp flanks might be insufficient to achieve homologous recombination. It was hoped that 2kb flanks would provide the necessary specificity for site-specific integration. The 2kb regions of DNA immediately upstream and downstream of the GPCR coding region were amplified by PCR using MHV-68 DNA as a template (pair 7 for left flank; pair 8 for right flank, appendix 1). The GFP gene was amplified (pair 17, appendix 1) using the pEGFP-C1 plasmid as a template (appendix 2). The PCR product included the CMV immediate early promoter, the GFP coding region and an SV40 polyadenylation site. The pEGFP-C1 multiple cloning site, which is situated at the 3' end of the GFP

Figure 3.5.1A The Homologous Recombination Event



The recombination cassette contains regions of DNA complementary to the sequences flanking the GPCR. Binding of cassette DNA to the MHV-68 genome results in a homologous recombination event that replaces the GPCR with the marker gene, GFP. The picture shows a GFP^+ virus plaque on a BHK-21 monolayer.

coding region, was excised prior to amplification, which assisted subsequent cloning of the GFP PCR product. The 2kb homologous flanking regions and the GFP gene were individually inserted into the pKS (-) plasmid in a three-step cloning strategy as depicted in figure 3.5.1B. The 5.4kb construct was excised from the plasmid using the restriction enzymes, *Xho*I and *Pvu*I, to avoid any recombination events leading to incorporation of the whole plasmid.

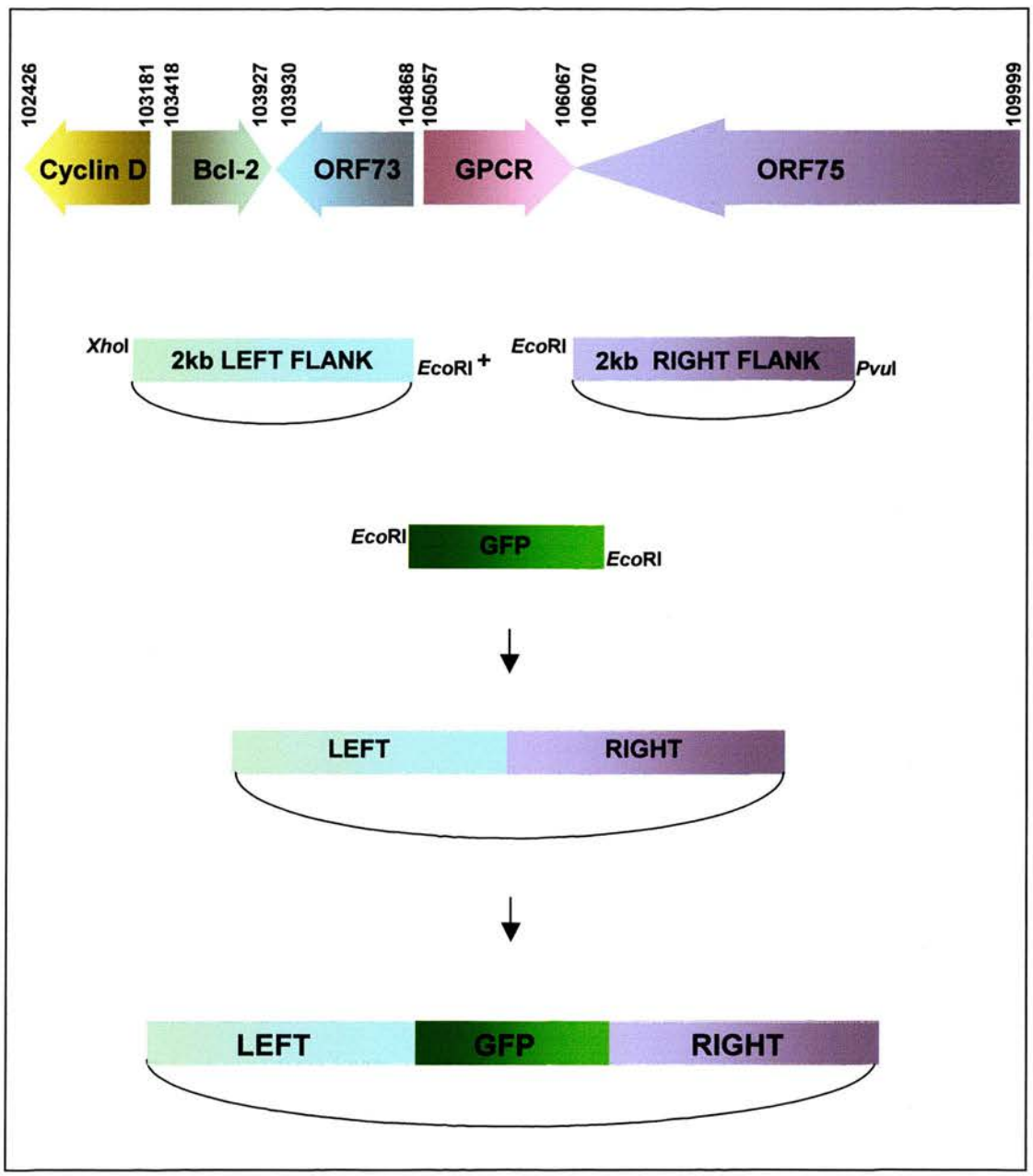
3.5.2. Recombination of DNA in Transfected Cells

To facilitate the recombination event, BHK-21 cells were co-transfected with the excised recombination cassette and MHV-68 DNA. Firstly, it was necessary to determine the amount of MHV-68 DNA required to produce a sufficient number of isolated infectious virus plaques. Two methods of transfection, electroporation (2.4.3) and lipofection (2.4.4), were used to compare the efficiency of DNA delivery. BHK-21 cells were transfected with various amounts of virus DNA (5, 10, 15, 20, 25, 30μg) and incubated for four days. The cells were fixed and stained and the number of plaques counted. It was shown that transfection of cells with 10μg of MHV-68 DNA resulted in an appropriate number of isolated infectious virus plaques. Electroporation proved to be a slightly more efficient method of transfection than lipofection and so electroporation was used throughout. Recombinant virus was generated as described in section 2.4.12. BHK-21 cells ($2x10^6$) were transfected with 10μg MHV-68 DNA and 10μg of recombination cassette. The cells were overlaid with agar 24 hours post-transfection. At four days post-transfection, wild type virus plaques were observed using the light microscope and plaques containing GFP^+ recombinant virus were visible using a UV microscope.

3.5.3. Purification of Recombinant Virus

Recombinant MHV-68 was isolated from wild type virus by plaque purification (2.4.13). Five GFP^+ plaques, that were well isolated from wild type plaques, were picked using a Pasteur pipette. BHK-21 cells were infected with virus that was extracted from each agarose plug. Four rounds of plaque purification were performed until only a few wild type plaques were observed. The recombinant virus was then purified using a limiting dilution assay (2.4.14). A GFP^+ plaque harvested

Figure 3.5.1B Design of the Recombination Cassette



Cloning strategy for production of a homologous recombination construct. The 2kb regions of DNA directly flanking the GPCR coding region were amplified by PCR and inserted into the cloning vector, pKS(-). The GFP coding region, under the control of the CMV promoter, was inserted between the left and right flanking regions in the pKS(-) vector. The primers used to amplify each fragment included unique restriction sites, not encoded on the fragments, to facilitate directional cloning. In the case of GFP, correct orientation of the gene was confirmed by restriction analysis. The 5.4kb recombination cassette was excised from the plasmid prior to transfection.

from the last round of plaque purification was propagated and titrated on BHK-21 cells to determine the concentration of virus required to produce a single plaque per well on a 96-well plate. C127 cells were used in this assay since MHV-68 forms more discrete plaques on this cell line than on BHK-21 cells. The virus was added to C127 cell monolayers in 96-well plates at a predicted concentration of 0.4pfu/well. Therefore, for every 10 wells infected, four wells should have contained a single plaque arising from a single plaque-forming unit of virus. A single GFP-expressing plaque was isolated and the virus was propagated for the extraction of DNA (2.2.19).

The recombinant virus DNA was analysed by Southern blotting (2.2.20). Both recombinant and wild type DNA was digested with three different restriction enzymes (*Bam*HI, *Bgl*II and *Eco*RI) and electrophoresed on four separate agarose gels alongside DNA molecular weight markers. The DNA was transferred onto membranes and the lanes bearing the markers were removed and stained (2.2.21). Each blot was hybridised with an [α ^{32}P]dCTP-labelled DNA probe encoding either the right flank, left flank, GFP or the GPCR (Figure 3.5.3A). On each blot, the wild type banding pattern is also evident in the recombinant digests. This indicates that the recombinant virus was contaminated with wild type virus despite the apparent absence of wild type plaques. Hybridisation with the left flank reveals an identical banding pattern in the recombinant and wild type DNA digests, which suggests that the left flank has been completely deleted. The closely spaced bands, or "laddering" effect, produced by hybridisation with either the right flank or GFP probe indicates proximity to a region of repetitive DNA, such as the terminal repeats. The number of terminal repeats varies in a heterogeneous population of virus thus producing the 1.2kb graduations in size of the bands (Efstathiou *et al.*, 1990b). The molecular weight of each band was calculated and the integration site of the cassette was determined by analysis of the MHV-68 restriction sites.

A restriction map was constructed from this data showing the approximate site of integration (Figure 3.5.3B.). The evidence suggests that a non-homologous recombination event occurred which resulted in integration and partial deletion of the cassette at the extreme left-hand region of the genome, proximal to the terminal

Figure 3.5.3A Southern Analysis of RCVI DNA

Southern blots of MHV-68 recombinant (RCVI) and wild type (WT) DNA. Virus DNA was digested with one of three restriction enzymes: *Eco*RI, *Bgl*II or *Bam*HI. RCVI DNA (5μg/lane) and WT DNA (3μg/lane) were electrophoresed on 1% agarose TAE gels alongside DNA molecular weight markers (5μg 1kb DNA ladder). The DNA was transferred to nylon membranes by capillary transfer and fixed by UV cross-linking. Each blot was probed with a different [α^{32}P]dCTP-labelled DNA probe corresponding either to the right flank (A), GFP (B), the left flank (C) or the GPCR (D). Blots were exposed to autoradiographic film for 24 hours. Bands corresponding to wild type virus DNA are shown in red and recombinant virus DNA in green.

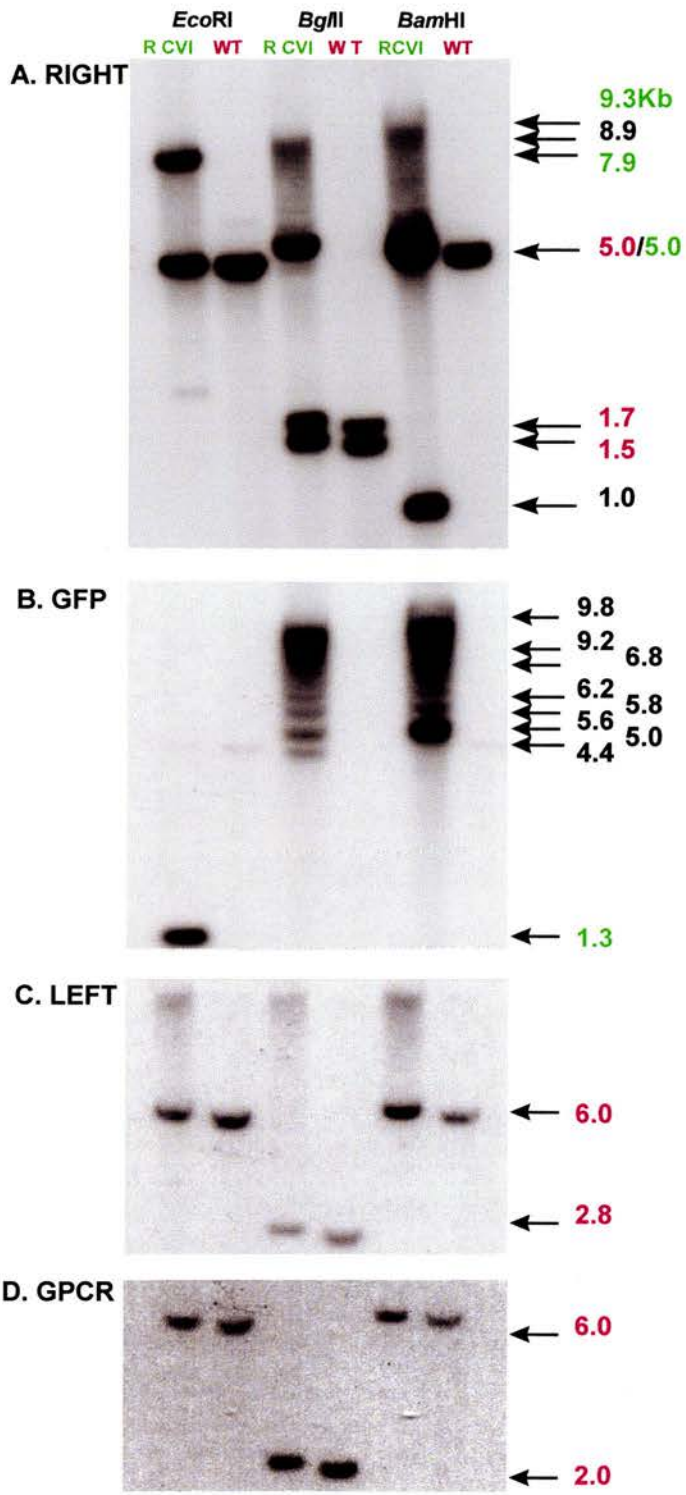
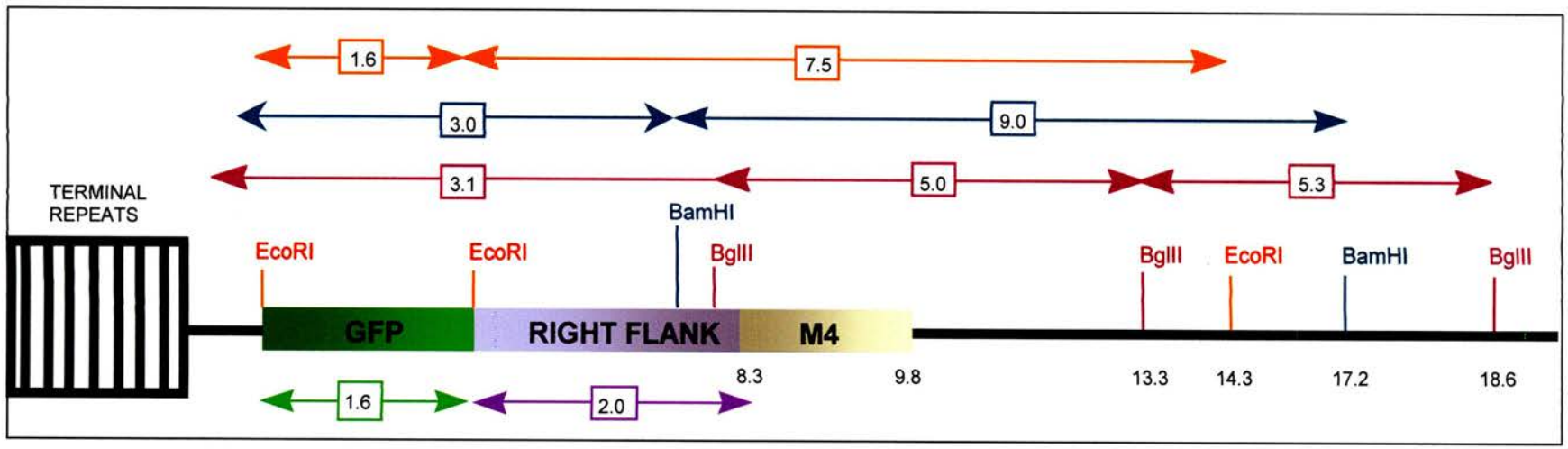


Figure 3.5.3B Restriction Map of RCVI



Restriction site map based on Southern analysis shown in figure 3.5.3A depicting the non-homologous recombination of the recombination cassette. Comparison of actual band sizes with those predicted from restriction analysis of the MHV-68 genome (Efstathiou, 1990b) showed that the construct inserted adjacently to the terminal repeats at the left-hand end of the MHV-68 genome. This resulted in partial deletion of the construct and approximately 8kb of the virus DNA including genes M1, M2 and M3.

repeats. There appears to have been a concomitant deletion of the virus genome between the repeat region and the integration site of the cassette. The relative sizes of the bands would indicate that the viral tRNAs and genes, M1, M2 and M3 have been deleted. Illegitimate recombination at the left-hand end of the MHV-68 genome has been shown to occur when using other recombinant constructs (Roy *et al*, unpublished observations). It is probable that this region of the genome contains a recombinational hot-spot. This region is a potential candidate for the virus origin of replication, which would have an effect on the genomic structure in this region. Active regions of DNA have a more open configuration that may increase the likelihood of recombination. It is possible that integration of the cassette also occurred at the correct site in addition to the non-specific site. However, since the deletion of the GPCR may have a deleterious effect on virus growth, it is possible that the illegitimate recombinant would have a selective advantage. In this case, selection for GFP-expressing plaques would be unlikely to yield recovery of the homologous recombinant.

3.5.4. Generation of a Recombinant virus - RCVII

A potential strategy to circumvent the problem of illegitimate recombination was to produce a construct with longer flanking sequences. Theoretically a construct containing flanking sequences in the region of 5kb would provide a greater level of specificity. The disadvantages of using longer flanks, however, were that the DNA fragments would be more difficult to amplify by PCR and the number of restriction sites within the 5kb sequences would preclude directional cloning. Moreover, it was unclear whether a larger cassette would be any less likely to recombine at the MHV-68 left-hand end hot-spot. Since the problem appeared to be the presence of the hot-spot rather the construct, it was desirable to use a target virus that did not contain this region.

MHV-76 is one of the original viruses isolated concurrently with MHV-68 (Blaskovic *et al.*, 1980). Limited sequence and restriction fragment length polymorphism (RFLP) analysis has shown that MHV-76 is likely to be a deletion variant of MHV-68 (Macrae, unpublished observations). MHV-76 appears to be

identical to MHV-68 except that it lacks four of the unique genes (M1-M4) at the left-hand end of the genome. Therefore, it seemed reasonable to conjecture that MHV-76 may also lack the recombination hot-spot. As the MHV-76 genome had not been fully sequenced, it was necessary to ensure that MHV-76 encoded a GPCR that was equivalent to the MHV-68 GPCR. The MHV-76 GPCR was amplified using the same primers designed for the MHV-68 GPCR and inserted into the cloning vector, pKS (-). The DNA sequence of the MHV-76 GPCR was determined using an automated sequencing machine (2.2.24) and found to be identical to the MHV-68 GPCR sequence. On the basis of this finding, it was hypothesised that deletion of this gene in either virus would yield a similar result.

Recombinant MHV-76 was generated in the same way as described for MHV-68. Five GFP^+ plaques were isolated post-transfection and each was grown at three dilutions (1:10, 1:100, 1:1000) in 6-well plates containing BHK-21 cells. After four days, the number of GFP^+ plaques was counted and then the cells were fixed and stained. By subtracting the number of GFP^+ plaques from the total number of plaques, it was possible to estimate the amount of wild type virus in each sample (Table 3.5.4). Virus sample number 1 had the lowest ratio of wild type virus to recombinant virus plaques and was therefore selected for further purification by Southern analysis. A PCR strategy was devised to test whether integration of the construct had occurred at the correct site (figure 3.5.5). Using a primer just outside the construct and a primer corresponding to GFP (pair 9, appendix 1), it was possible to amplify across the region of the insertion. The PCR product was produced only if the construct containing GFP had integrated correctly. Using the same upper primer and one corresponding to the GPCR (pair 10, appendix 1) it was possible to amplify a similarly sized region of wild type virus DNA. This provided a method of testing whether wild type virus was still present in each sample and also controlled for the quality of the DNA.

3.5.5. Analysis of RCVII by PCR

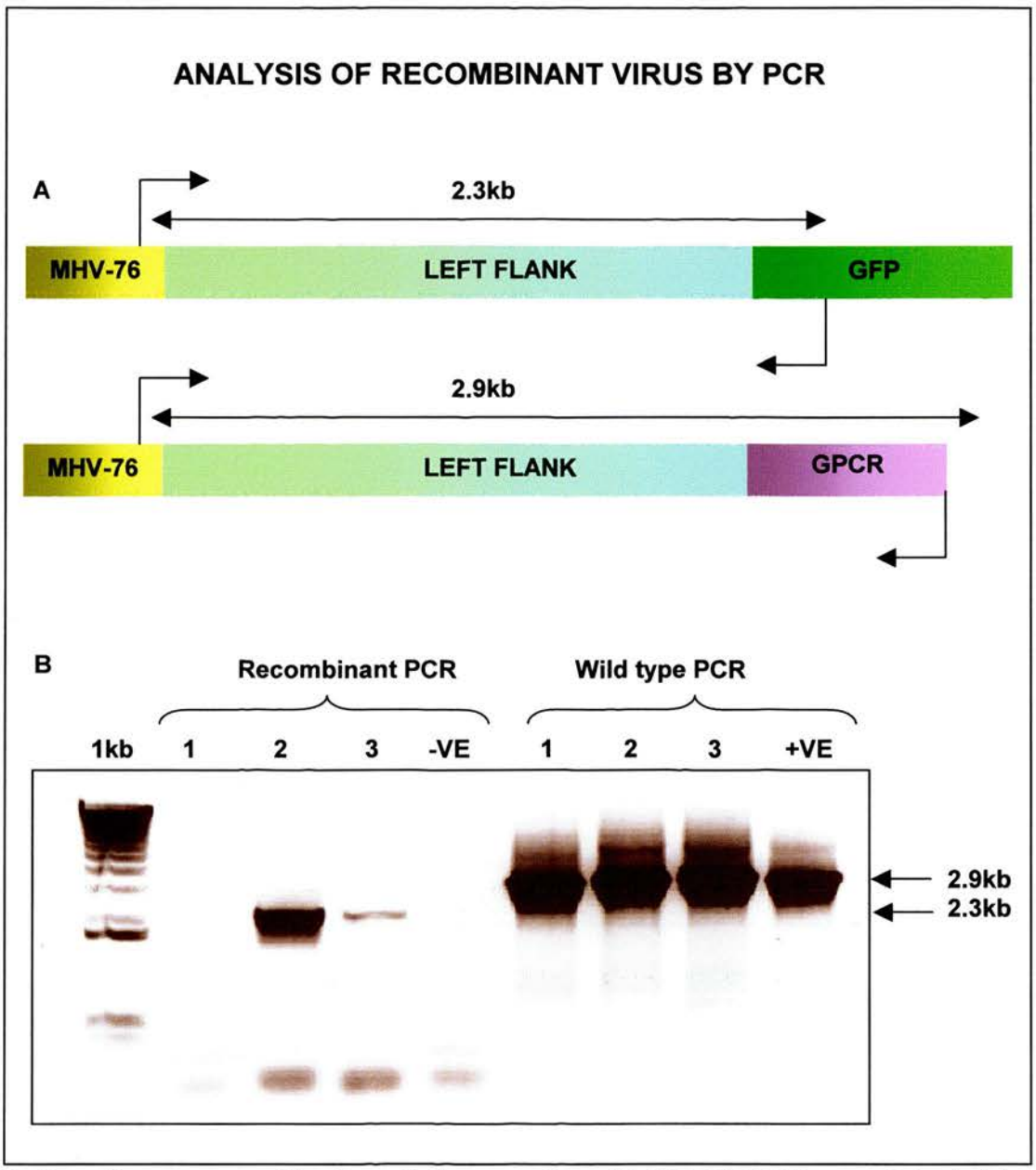
Before carrying out further rounds of purification of the recombinant virus, it was advantageous to examine whether homologous recombination had occurred.

Table 3.5.4. Ratio of Recombinant to Wild Type Virus

VIRUS SAMPLE	DILUTION FACTOR	GFP$^+$ PLAQUES	TOTAL PLAQUES	WT > RCVII
1	10^{-1}	-	-	6X
	10^{-2}	92	-	
	10^{-3}	11	61	
2	10^{-1}	476	-	8X
	10^{-2}	60	-	
	10^{-3}	5	43	
3	10^{-1}	265	-	22X
	10^{-2}	14	-	
	10^{-3}	2	45	
4	10^{-1}	63	-	31X
	10^{-2}	8	-	
	10^{-3}	1	31	
5	10^{-1}	-	-	6X
	10^{-2}	418	-	
	10^{-3}	30	174	

The number of GFP$^+$ and wild type plaques were compared at each titration to determine the ratio of recombinant (RCVII) to wild type (WT) virus. The dashed lines indicate that the CPE was too great to estimate the number of individual plaques.

Figure 3.5.5. PCR Analysis of Recombinant Virus



A. Homologous recombination of the cassette with MHV-76 DNA was detected by PCR. Amplification across the insertion produced a band of 2.3kb. A PCR product of similar size (2.9kb) was produced by amplifying the non-recombined region. This served as a positive control for the quality of the virus DNA and also detected the presence of wild type virus. B. Three virus clones expressing GFP were isolated by plaque purification and assayed for the presence of recombinant virus. Recombinant virus is only detectable in samples 2 and 3 whereas all three are contaminated with wild type virus. The negative control PCR contained no DNA while the positive control template was purified MHV-76 DNA.

Southern analysis was performed as previously described for RCVI on all five of the virus samples. The data, which was not photographically reproducible, suggested that sample no.1 contained virus that had undergone homologous recombination. However, the ratio of wild type virus in the sample made it difficult to confirm this Ten plaques, generated by sample no. 1 in the next round of plaque purification, were selected and propagated in 24-well plates. The DNA was extracted using a kit (QIAmp®DNA Mini Kit, QIAGEN) as described in section 2.2.18. The DNA (10µl/reaction) was amplified by PCR using *Taq* polymerase. All ten samples tested positively for wild type virus and two samples contained recombinant virus. Following two subsequent rounds of plaque purification, all samples remained positive for wild type virus and only two samples were positive for the recombinant genotype. Plaque purification did not yield pure recombinant virus.

3.5.6. Plaque Purification using a Complementary Cell Clone

The failure to isolate recombinant virus by plaque purification indicated that the GPCR might be essential to virus replication and that deletion of the gene had severely debilitated the virus. In this scenario, the recombinant virus might require the presence of the wild type virus to complement the mutation. It might also be possible to complement the mutation by propagating the recombinant virus in cells that expressed the GPCR. Therefore, plaque purification was carried out using stably transfected 3T3 cells expressing the GPCR (GPCR1, see section 3.4.3). A virus sample that tested positively for recombinant virus by PCR in the previous round of plaque purification was grown on $GPCR^+$ cells. In contrast to BHK-21 cells and C127 cells, the virus did not infect the 3T3 clone efficiently. Only three plaques were recovered and tested by PCR: all tested positive for wild type virus and only two plaques contained recombinant virus (figure 3.5.5B.). Thus the complementary cell line did not appear to resolve the purification problem.

3.4.7. Growth of RCVII in B cells

Murine gammaherpesvirus, like EBV, establishes latency in B cells. There is evidence to suggest that passage of EBV^+ B cell lines in culture results in the predominance of a single latent EBV episome, as determined by the lack of variation

in the numbers of terminal repeats (Kintner & Sugden, 1981). The resistance of EBV-infected cell lines to superinfection also indicates that each B cell supports only one episomal genomic variant (Reisman & Sugden, 1984). On the basis of this finding, a possible purification strategy appeared to be to infect B cells with the mixed population of wild type and recombinant virus and clone individual cells carrying either wild type or recombinant latent virus. The non-secreting-immunoglobulin B cell line, NS0 (Kholer, 1976), was used in this experiment. NS0 cells ($5x10^5$) were infected with recombinant virus isolated from the last round of plaque purification (virus sample no.3, figure 3.5.5B) in a volume of 100µl for 1 hour at 37°C. The cells were transferred to a T25cm^2 flask and propagated for several days to ensure that all the cells had become infected. The infected cells were cloned using a limiting dilution method. Cells ($1x10^5$) were serially diluted in 96-well plates containing mixed thymocyte medium to a concentration that would give rise to a single B cell. No single B cells expressing GFP were observed. Attempts were made to enrich for B cells expressing GFP by selectively pooling GFP$^+$ cells. However, the expression of GFP appeared to be unstable, indicating either that the B cells infected with recombinant virus were not surviving or that GFP was not expressed during virus latency. Therefore, it was not possible to clone a single B cell expressing GFP.

Instead, the infected B cells were used in an infective centre assay (2.4.11), which involves co-cultivation of latently infected cells with BHK-21 cells, resulting in reactivation of virus from single cells. The infected B cells were incubated with the anti-herpetic drug, 2'-deoxy-5- ethyl-beta-4'thiouridine (C9), for 7 days to suppress lytic replication prior to overnight co-cultivation with BHK-21 cells. The following morning, the medium was removed and the BHK-21 cells were overlaid with agar. No wild type plaques were observed, only plaques expressing GFP. Thirty plaques expressing GFP were selected and analysed by PCR. All the virus samples contained both recombinant and wild type virus.

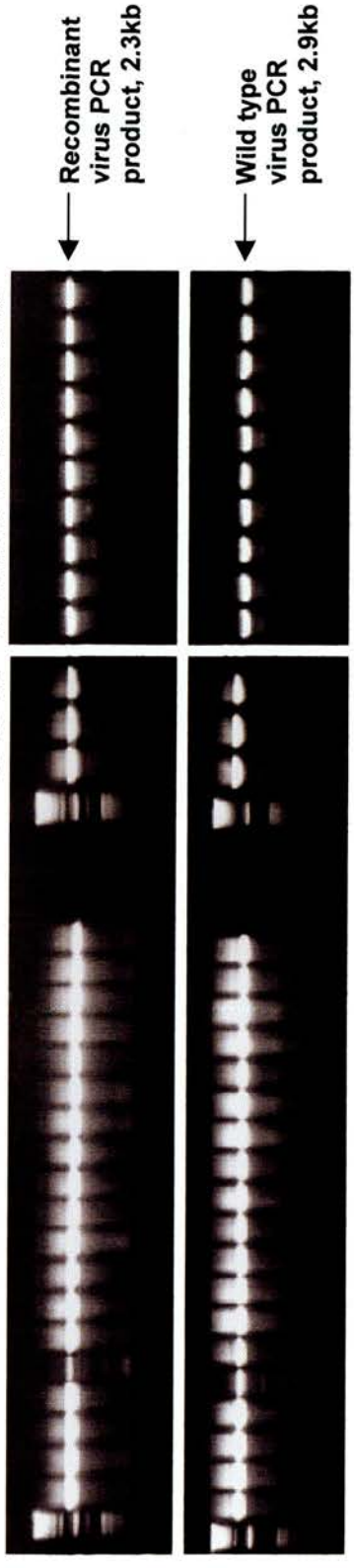
3.5.8. Infection of Mice with Recombinant Virus

The final purification strategy involved infection of mice with the contaminated recombinant virus. There was evidence showing that splenic B cells harvested from mice infected with a mixed population of virus reactivated homogeneous virus in infective centre assays (Macrae, unpublished). Virus sample no.3 (figure 3.5.5B) was propagated (2.4.9) and titrated (2.4.10) to determine the concentration. Four mice were infected intranasally with virus: two received $1x10^2$ pfu and two received $1x10^3$ pfu (2.4.15.). Transgenic mice lacking the α/β interferon receptor were used in this experiment, as they do not resolve acute MHV infection as rapidly as wild type mice (Dutia *et al.*, 1999). Since MHV-76 is less virulent than MHV-68 in wild type mice, it was necessary to ensure that the mice yielded sufficient latently infected splenocytes by using the more susceptible mice. The spleen of each mouse was harvested on day 14 post-infection when splenomegaly is at its peak. Infectious virus was reactivated from the splenocytes in the same way as described above. Again, no wild type virus plaques were observed and thirty isolated GFP^+ virus plaques were selected. PCR analysis showed that all the plaques contained both wild type and recombinant virus (figure 3.5.8).

3.5.9. Southern Analysis of RCVII

At this point, the recombinant virus had been purified to the standard that no wild type plaques were visible under the light microscope. Therefore, it was more likely that Southern analysis could now be carried out to confirm correct integration of the recombination cassette. Virus sample no.3 was propagated and DNA was extracted for Southern analysis as previously described for RCVI. As before, restriction maps were constructed showing the expected banding pattern for each probe (figure 3.5.9A). The results of the Southern analysis are shown in figure 3.5.9B. As was the case for RCVI, the recombinant banding pattern also contained bands that corresponded to the wild type virus thus confirming that this virus sample was a mixed population. There was also a "laddering" effect detected by probes for the right flank and GFP, which indicates that this population of viruses contains an illegitimate recombinant. The appearance of large bands in all restriction digests,

Figure 3.5.8. Co-purification of RCVII and WT MHV-76 from Infected Mice

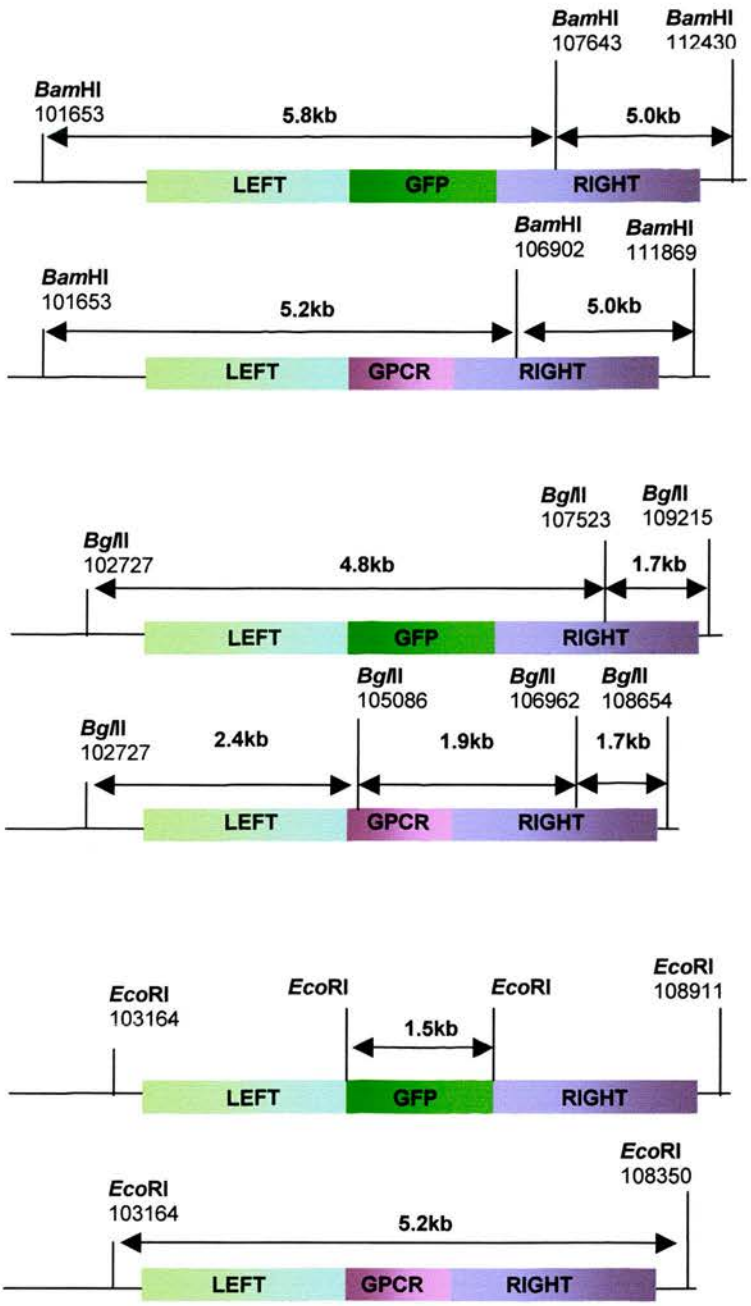


Purification of recombinant virus. Four mice were infected with either $1x10^2$ or $1x10^3$ pfu of a mixed population of recombinant and wild type MHV-76. The spleen of each mouse was harvested at 14 days post-infection and the splenocytes were co-cultivated with BHK-21 cells. Reactivation of latent virus resulted in the formation of GFP^+ plaques that were isolated as described for the plaque-purification assay. Virus was propagated from each plaque and the DNA analysed by PCR for the presence of wild type and recombinant MHV-76. The PCR products ($^1/_{10}$ sample) were electrophoresed on a 0.8% agarose TAE gel. Each virus plaque tested positively for both wild type and recombinant virus.

especially *Eco*RI, indicates that partial digestion has occurred. However, close examination of the recombinant virus DNA banding pattern reveals the presence of faint bands that correspond to the expected size. This verifies the PCR data showing that the cassette has integrated correctly.

Interestingly, the Southern data also raises the possibility that the recombinant virus is not contaminated with wild type virus but with an illegitimate recombinant. This would explain why it was apparently impossible to purify the virus by selecting for GFP-expressing virus plaques.

Figure 3.5.9A Restriction Maps of RCVII and WT MHV-76 DNA



***Bam*HI BAND SIZES (kb)**

PROBE	RCVII	WT
LEFT:	5.8	5.2
RIGHT:	5.8/5.0	5.2/5.0
GFP:	5.8	-
GPCR:	-	5.2

***Bgl*II Band Sizes (kb)**

PROBE	RCVII	WT
LEFT:	4.8	2.4
RIGHT:	4.8/1.7	1.9/1.7
GFP:	4.8kb	-
GPCR:	-	2.4/1.9

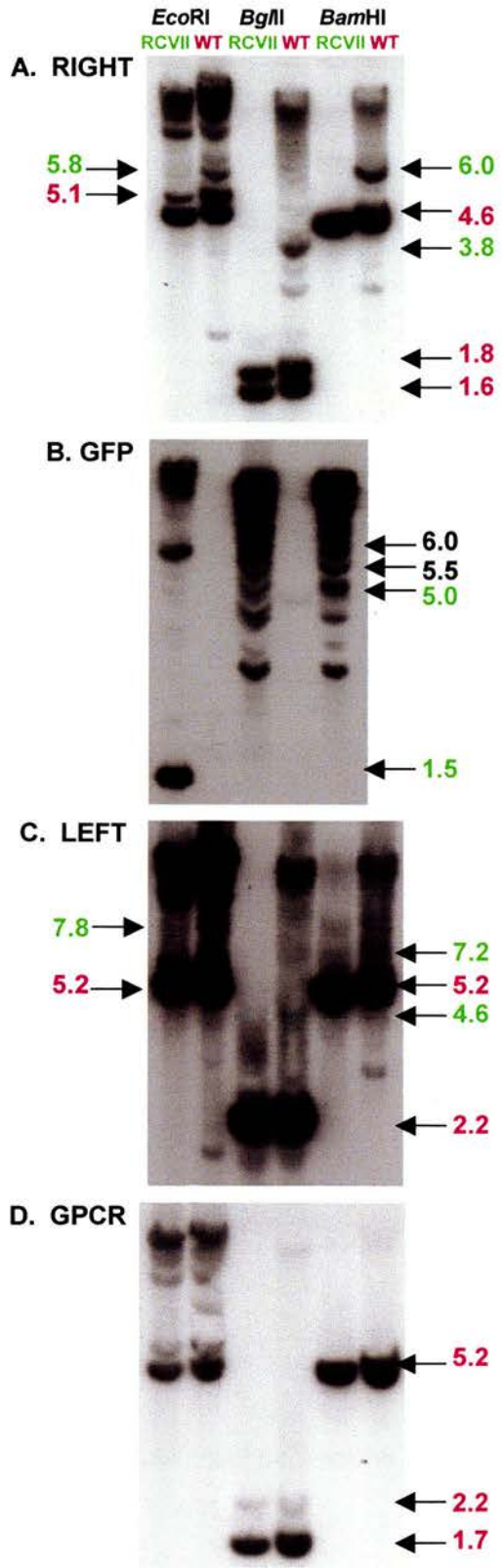
***Eco*RI Band Sizes (kb)**

PROBE	RCVII	WT
LEFT:	5.8	5.2
RIGHT:	5.8	5.2
GFP:	1.5	-
GPCR:	-	5.2

Restriction maps of the murine gammaherpesvirus genome in the region surrounding the GPCR. Mapping data was obtained using the gcgv.10 sequence analysis programme (Devereux *et al.*, 1984). MHV-68 genome co-ordinates are given according to (Virgin *et al.*, 1997). The predicted band sizes are shown for restriction digests of wild type and recombinant virus DNA digested with *Bam*HI, *Bgl*II or *Eco*RI and detected using probes for the right flank, left flank, GFP or the GPCR in Southern analysis.

Figure 3.5.9B Southern Analysis of RCVII DNA

Recombinant (RCVII) and wild type (WT) MHV-76 DNA were digested with one of three restriction enzymes: *Eco*RI, *Bgl*II or *Bam*HI. Digested RCVII DNA (5μg/lane) and WT DNA (3μg/lane) were electrophoresed alongside DNA molecular weight markers (1kb ladder, 5μg/lane) on four 1% agarose TAE gels. The DNA was transferred to nylon membranes by capillary transfer and fixed to the blot by UV cross-linking. Each blot was hybridised with a different [α^{32}P]dCTP-labelled DNA probe corresponding either to the right flank (A), GFP (B), the left flank (C) or the GPCR (D). The blots were exposed to autoradiographic film for 1-2 hours. The size of each band was calculated by reference to the molecular weight markers. Bands corresponding to RCVII DNA are shown in green while WT bands are shown in red.



Chapter Four: Discussion

G protein-coupled receptors are among the many examples of cellular proteins that have been acquired by viruses, a phenomenon that has been termed "molecular piracy". Although there is little sequence conservation between viral homologues, the presence of GPCRs in so many virus genomes indicates that these receptors confer a selective advantage. Several approaches have been taken to investigate the role of the MHV-68 GPCR including transcriptional analysis, expression of the protein and an examination of the potential oncogenicity of the receptor. In order to evaluate the individual contribution of this gene to viral pathogenesis, attempts were made to generate a recombinant virus lacking the GPCR.

4.1. Sequence Analysis of ORF74

Various programmes were used to predict that the MHV-68 ORF74 sequence contains motifs characteristic of a G protein-coupled receptor. Alignment of viral GPCRs with mammalian CXCR2 and the EBV-induced protein, CCR7, demonstrated the variation not only between the viral homologues and the mammalian chemokine receptors but also between the viral genes themselves. It is clear, however, that the MHV-68 GPCR bears more similarity to the KSHV and HVS GPCRs than to the AHV-1 GPCR, which is not a positional homologue.

In terms of the DRY motif, the MHV-68 sequence (HRC) appears to most closely resemble HVS, which encodes LRC at this position. However, the aspartic acid residue is considered to be the most critical amino acid in the DRY motif as naturally occurring and engineered substitutions of this residue engenders constitutive GPCR signalling (Alewijnse *et al.*, 2000; Burger *et al.*, 1999). It has been hypothesised that replacing the aspartate with a hydrophobic residue such as valine would draw the intracytoplasmic loop into the plasma membrane, rendering the GPCR in a permanently active conformation. Since leucine, like valine is a highly hydrophobic residue, the D→L change bears similarity to the KSHV D→V change. This implies that the HVS GPCR may be constitutively active, yet the induction of calcium mobilisation in response to chemokines suggests that signalling is actually ligand-dependent (Ahuja & Murphy, 1993). Histidine is a basic amino acid that is unlikely to be buried in the plasma membrane but since a hydrophobic residue does not

appear to automatically confer agonist-independence, the MHV-68 HRC motif is not a reliable indicator of agonist-dependent or independent signalling activity. The transforming activity of the MHV-68 GPCR did not appear to be dependent on chemokine stimulation, although there may have been agonists present in the medium that could have activated the receptor. Therefore, it would be interesting to examine the signalling activity of the GPCR in response to a range of potential chemokine agonists.

4.2. Transcription Pattern of the GPCR

4.2.1. Transcription of the GPCR during Productive Infection

The transcriptional expression pattern of the GPCR during productive infection *in vitro* was examined by Northern hybridisation. This revealed the presence of four GPCR-specific transcripts that were only barely detectable using this technique. This indicates that the gene is expressed at very low abundance and coincides with the observations of Virgin *et al.*, 1999, who failed to identify GPCR transcripts in productively infected fibroblasts. It is also in keeping with analysis of the KSHV GPCR, which has shown that it is expressed at low levels in KS lesions (Kirshner *et al.*, 1999).

The kinetic class of the GPCR transcripts was established by treating the cells with chemical inhibitors. GPCR transcription was resistant to treatment with phosphonoacetic acid (PAA) and acyclovir (ACV), which both inhibit herpesvirus DNA replication. Since expression of the late kinetic class of genes occurs only after virus replication, this indicates that the GPCR is an immediate early or an early gene. The sensitivity of GPCR transcription to cycloheximide, which suppresses all but immediate early gene expression, could not be assessed as GPCR-specific transcripts were not evident prior to 12 hours post-infection (p.i.). Since MHV-68 early genes are generally visible by 8 hours p.i. and late genes by 12 hours post-infection (Stewart *et al.*, 1996; Stewart *et al.*, 1994), this in itself indicates that the GPCR is unlikely to be an immediate early gene.

The apparent down regulation of GPCR transcription in the presence of PAA is possibly a result of the toxic effect of PAA on the BHK cells. An alternative explanation is that the GPCR is expressed at both early and late time-points and that PAA is suppressing transcription at late time-points. If this were the case, a similar down regulation might be expected with ACV treatment although the transcription of gp150 in the presence of ACV has demonstrated a degree of leakage through this block. Previous analysis has indicated that ACV is a less potent inhibitor of MHV-68 than a related nucleoside analogue, C9 (2'-Deoxy-5-ethyl-beta-4'-thiouridine, (Barnes *et al.*, 1999).

The designation of the GPCR as an early-leaky-late lytic gene corresponds to the expression pattern of the KSHV GPCR. The induction of productive KSHV infection with phorbol esters in latently infected cell lines has revealed that GPCR transcription is resistant to PAA but peaks later than other classic early genes (Kirshner *et al.*, 1999; Sun *et al.*, 1999). KSHV GPCR expression is barely detectable in un-induced $KSHV^+$ PEL cells lines but since these lines undergo a low level of productive replication, it is likely that the GPCR transcripts arise from cells in the productive cycle (Cesarman *et al.*, 1996b). This is confirmed by *in situ* hybridisation analysis of KS lesion showing co-localisation of GPCR transcripts with lytic cycle genes (Kirshner *et al.*, 1999).

4.2.2. Latent Expression of the GPCR

Expression of the GPCR could not be detected in S11 cells using Northern analysis. As discussed in section 1.5, S11 cells are latently infected with MHV-68 but a low percentage of cells (1-2%) undergo lytic replication that can be suppressed with ACV or C9 (Usherwood *et al.*, 1996c). Similarly to the KSHV and EBV latently infected cell lines, productive replication can be induced by stimulation with phorbol ester. GPCR-specific transcripts were not evident in Northern hybridisations of S11 cells or S11 cells treated with the phorbol ester, TPA. This may reflect the sensitivity of the technique and the observation that only a proportion of S11 cells are reactivated by TPA, whereas *in vitro* infection of a fibroblast population with a high multiplicity of infection is likely to result in the infection of every cell.

However, using Southern analysis, it has been shown that cDNA derived from S11 cells hybridises with genomic DNA corresponding to ORF74 and that this effect can be abrogated by treating the cells with C9 (Simas *et al.*, 1999). This indicates that in S11 cells at least, the GPCR is not transcribed during latency. As with EBV, there are apparent differences between the *in vitro* model of latent infection and the latency-associated patterns of gene expression observed *in vivo*.

Using RT-PCR, transcripts corresponding to the region of the genome encoding the GPCR were detected in the lungs of mice at 10 months p.i. with MHV-68. Since this experiment was carried out using random primed cDNA and double-stranded DNA probes, the signals detected may not be specific for the GPCR or v-Bcl-2. Transcripts specific for ORF73, a gene transcribed in the opposite direction, may also encode the GPCR and v-Bcl-2. The KSHV homologue of ORF73 encodes the LANA protein, which has been shown to be latency-associated. Therefore, it is possible that the positive signals detected in this experiment actually represent expression of ORF73. Clarification of this point could be achieved by repetition of this experiment using gene-specific primers for the reverse transcription reaction and single-stranded riboprobes to detect the PCR products.

Previous analysis identified both linear and episomal MHV-68 genomes in the lung at late time-points post-infection, implying the presence of latently and productively infected cells (Stewart *et al.*, 1998). Transcription of the GPCR in the absence of classic lytic cycle genes such as the immediate early gene, ORF50, would be indicative of expression in latently infected cells. GPCR expression has also been observed in latently infected peritoneal exudate cells (PECs) without concurrent transcription of lytic genes (Virgin *et al.*, 1999). Latency-associated expression of the GPCR was detected in the spleen of one mouse (1 of 3) at 10 months p.i. This corresponds to the study by Virgin *et al.*, in which GPCR transcription was observed in the spleens of a small proportion of infected mice (2 of 16). Since the virus may establish latency in a variety of cell types, it is reasonable to speculate that latent gene expression may be regulated differently in these organs.

4.2.3. Mapping of the GPCR Transcripts

The size of the GPCR transcripts suggested that they might be polycistronic. Polycistronic transcripts are not an uncommon feature in gammaherpesviruses; in fact the KSHV GPCR is transcribed bicistronically with the KSHV homologue of the integrin-like adhesion molecule, Ox-2 (Talbot *et al.*, 1999). Other examples include the KSHV v-cyclin and v-FLIP genes (Yu *et al.*, 1999), the rat CMV (RCMV) GPCR which is encoded on a bicistronic transcript (Beisser *et al.*, 1998) and the HVS dihydrofolate reductase transcript, which encodes two other unknown ORFs and five small RNA molecules (Whitaker *et al.*, 1995). Using RT-PCR, a transcript was identified that appears to encode the MHV-68 v-Bcl-2 and the GPCR. Unlike the KSHV GPCR-v-Ox-2 transcript, the MHV-68 v-Bcl-2-GPCR transcript does not appear to be spliced. The co-expression of GPCR and v-Bcl-2 is supported by the *in vivo* RT-PCR data, which suggests an identical pattern of transcription of the GPCR and v-Bcl-2 in the lungs and spleen of every mouse.

Analysis of the 3' end of the GPCR transcript using 3'RACE suggested that it terminates at the only available consensus polyadenylation signal (AAUAAA) approximately 600bp downstream of the GPCR translational STOP site. Although the RT-PCR reaction generated multiple GPCR-specific bands, the smaller products do not correspond to polyadenylation sites and most likely arose from mis-priming of the oligo(dT) primer at several potential AT-rich regions within the GPCR coding region. As a low annealing temperature was essential for this amplification, the stringency of the reaction could not be increased. Confirmation that this is indeed the 3' end of the transcript would require cloning and sequencing of the PCR product. However, when considered alongside the RT-PCR data showing expression of v-Bcl-2 and GPCR on the same transcript, the evidence suggests that the 3.4kb band detected by Northern hybridisation may represent a bicistronic transcript encoding the GPCR and v-Bcl-2. The identity of the other Northern transcripts is unknown. The appearance of less abundant, higher molecular weight transcripts has also been observed in Northern analysis of KSHV genes including vMIPI, GPCR, v-Bcl-2 and the capsid protein, ORF26 (Yu *et al.*, 1999). It is possible that they represent unspliced variants or readthrough transcription past the polyadenylation

signal into the 3' untranslated region. However, this could be elucidated by further Northern hybridisation with probes corresponding to genes in the region of the GPCR such as v-cyclin, v-Bcl-2 and ORF75. Alternatively, they may represent transcripts running in the opposite direction. Since the Northern blot was hybridised with a double-stranded DNA probe, it is possible that some of the transcripts originate from the complementary DNA strand and are not specific for ORF74. Many of the genes in the region of ORF74 are transcribed in the opposite direction, including ORF75, ORF73 and ORF72. This could be resolved by repeating the Northern hybridisation with an antisense riboprobe specific for the GPCR coding region, which would only hybridise with message from the sense strand of the genome.

The investigation of the 5' untranslated region of the transcript using 5'RACE did not yield conclusive results, as the major PCR product fell short of the GPCR translation initiation site. This was most likely due to premature termination of the reverse transcriptase enzyme, which can occur at RNA secondary structures. An alternative approach to circumvent this problem might be to attempt S1 nuclease mapping, a technique that does not involve reverse transcriptase but is nevertheless much less sensitive. Although the 5'RACE experiment generated multiple PCR products, none of them were of sufficient molecular weight to represent the v-Bcl-2-GPCR transcript. It is, however, possible that one of the minor products could represent a monocistronic GPCR transcript. If this were the case, the PCR reaction would favour a shorter cDNA template, which might also explain the absence of the longer bicistronic transcript. However, this seems unlikely as no monocistronic GPCR transcript was detected using Northern hybridisation, although it could be beyond the sensitivity threshold of the Northern analysis. Nevertheless, others have failed to find a monocistronic transcript encoding the KSHV GPCR (Kirshner *et al.*, 1999), which may provide an insight into the biology of these genes.

The KSHV GPCR may be translated from an internal ribosome entry site (IRES), which is an inefficient method of translation (Bieleski & Talbot, 2001). However, as the KSHV GPCR is a constitutively active signalling molecule (Arvanitakis *et al.*,

1997), a low level of expression may be sufficient for its function and higher levels may be detrimental. Overexpression of the gene *in vitro* has been found to trigger cell death, therefore KSHV may employ a translational regulation strategy to suppress expression of a potentially toxic protein (Kirshner *et al.*, 1999). In the case of MHV-68, the low abundance of GPCR-derived transcripts suggests that a similar mechanism may operate. The evidence indicates that the MHV-68 GPCR is a toxic gene: insertion of the GPCR gene into expression vectors was a difficult process, positive clones grew slowly and were subject to rearrangement; also, the transfection efficiency of GPCR expression constructs into eukaryotic cells was very much lower than control plasmids and produced fewer viable cells. The toxicity in bacteria may be caused by low-level readthrough transcription from cryptic promoters (Brown & Campbell, 1993). Expression of eukaryotic proteins can have a deleterious affect on prokaryotic cells, often causing problems in protein export machinery (Martin *et al.*, 1989). Overexpression of the GPCR in mammalian cells may induce apoptosis, however, it would be necessary to confirm this by carrying out TUNEL staining of transfected cells. Since the electroporation process itself results in a significant amount of cell death, it may be more informative to use an inducible expression system and stain stably transfected cells. It is perhaps not coincidental that the GPCR is co-transcribed *in vitro* and *in vivo* with v-Bcl-2. Expression of this anti-apoptotic gene may be required to overcome the toxicity of the GPCR by suppressing apoptosis

4.3. Expression of the GPCR Protein

The apparent toxicity of the GPCR protein has presented a problem in several aspects of this project: raising and testing an antibody against the GPCR, investigating the subcellular localisation of the receptor and determining whether it binds IL-8. All these experiments require a certain level of protein expression to facilitate recognition by an antibody or for the binding of radioactive ligand. There may be additional reasons for the low levels of expression, however. For instance, mutagenesis of the histamine H(2) receptor DRY motif resulted in structural instability of the GPCR. Since the MHV-68 GPCR does not encode a classical DRY motif, this may have implications for its stability.

4.3.1. Generation of an Antibody to the GPCR

As discussed in section 1.7, GPCRs are comprised of seven hydrophobic transmembrane domains connected by hydrophilic loops. Hydrophobic regions are not exposed on the cell surface and are therefore less likely to generate antisera that would recognise the native protein. For this reason, only the predicted N-terminal region of the GPCR, as defined by analysis of the hydropathy plot, was used as an antigen. In hindsight, this probably avoided a potential toxicity problem and produced a soluble antigen, which theoretically should have been easier to purify.

The GPCR N-terminus was expressed as a GST-fusion protein using the pGEX bacterial expression system. Inducing expression in bacteria resulted in a protein of the expected size that was recognised by an anti-GST antibody. However, an anti-MHV-68 antibody failed to recognise the fusion protein. Initially this suggested that the N-terminus might not be immunogenic, however it was reasoned that the GPCR might not generate a significant immune response during MHV-68 infection. Northern analysis showed that the GPCR is transcribed at barely detectable levels during productive infection and although the level of transcription does not necessarily correlate with the amount of protein translated, it is possible that there was insufficient GPCR protein to generate an immune response.

An affinity purification strategy was used to isolate the fusion protein, which was present at relatively low levels in the soluble fraction. However, the fusion protein bound glutathione-sepharose beads strongly and was resistant to elution with the recommended buffers. The residual presence of the fusion protein in the insoluble fraction indicated that the protein was only partially soluble, which may explain why it was refractory to elution in hydrophilic solutions. Nevertheless, the protein-bead complex was used as an antigen to raise antisera in rabbits.

The antisera were tested against Western blots of BHK cells that were productively infected with MHV-68 and a stable 3T3 cell clone that had been shown to express the GPCR using RT-PCR. Despite repeated immunisations, no differences were observed between the antisera and the pre-immune sera. This may reflect the low-

level expression of the GPCR protein in both these samples and the sensitivity of the Western analysis. However, the failure to detect a specific signal using the more sensitive radioimmunoprecipitation assay, suggests either the fusion protein was not immunogenic, or that antibody to the N-terminus did not recognise the native GPCR protein. Both antisera recognised the fusion protein on a Western blot, which confirmed that a humoural immune response was successfully raised against the antigen, but not whether it was specific for the GPCR N-terminus as opposed to GST. It is quite possible that this region of the GPCR had folded differently in isolation from the rest of the protein or that it was not fully exposed on the surface of the fusion protein. Alternatively, antibody against the N-terminus may not necessarily bind to the native GPCR. The principal caveat in using a bacterial system to express a eukaryotic protein is the lack of post-translational modification and the possibility of incorrect folding in the absence of eukaryotic molecular chaperones. Sequence analysis of the GPCR amino acid sequence predicted the presence of two glycosylation sites within the N-terminal region. Presumably, lack of glycosylation would affect the tertiary structure of the protein and hence the potential epitope.

An alternative strategy was to use DNA vaccination and synthetic peptides to stimulate an antibody response to the GPCR. The use of naked nucleic acid as a vaccine has generated much interest in the preceding decade and many reports attest to its efficacy in stimulating immune responses *in vivo* (Rothel *et al.*, 1997; Vogel & Sarver, 1995). The generation of an immune response using synthetic peptides has also been demonstrated (Nardin *et al.*, 2000), and is of particular value in situations where native protein antigens cannot be effectively expressed. In this case, sheep were chosen as the experimental animal since they are inexpensive and being larger than rabbits, they produce more serum. One sheep was also immunised with plasmid DNA encoding the cytokine, GMCSF, which is a growth factor for dendritic cells. Since most DNA vaccination is directed towards the skin, dendritic cells are the primary antigen-presenting cells involved in generating the immune response. The use of GMCSF as an immunological adjuvant is therefore an interesting hypothesis.

Neither sheep appeared to mount an immune response to the GPCR. As with the rabbit antisera, no specific recognition was observed using Western blotting and immunisation with the GMCSF DNA did not make any apparent difference. Again it must be pointed out that there may have been insufficient GPCR protein expressed in the transiently transfected cells to elicit a positive signal on the Western blot. Another potential caveat is that an immune response directed against synthetic peptides may not necessarily recognise the native protein.

4.3.2. Subcellular Localisation of the GPCR

To overcome the lack of anti-GPCR antibody, the subcellular localisation of the protein was determined using an "epitope-tagging" strategy. Immunofluorescence experiments detected expression of the GPCR in transiently transfected cells using the pVR1255-GPCRHA3 construct but not the pBabe-GPCRHA construct. Aside from having two extra haemagglutinin epitopes, it is not absolutely clear why expression was detectable using pVR1255 as opposed to pBabe/*puro*. It is also puzzling why an apparently toxic gene is expressed at all under the control of the powerful CMV promoter. However, others have used this plasmid successfully to express MHV-68 proteins that could not be expressed in alternative vectors (Atkin, 2000). The pVR1255 vector was systematically modified to optimise protein expression using a luciferase reporter system (Hartikka *et al.*, 1996). It contains the CMV immediate early intron A immediately upstream of the insertion site (see diagram 3.3.5A). This element, which has previously been shown to enhance gene expression in plasmid vectors (Chapman *et al.*, 1991), encodes binding sites for the transcription factor, nuclear factor 1, and enhanced luciferase expression by 25% (Hartikka *et al.*, 1996). Substitution of the bovine growth hormone polyadenylation signal for a minimal transcriptional terminator derived from the rabbit β globin gene (Levitt *et al.*, 1989) also doubled the expression of luciferase. Surprisingly, it was also found that replacing the ampicillin resistance gene with the kanamycin resistance gene significantly enhanced protein expression in a range of vectors (Hartikka *et al.*, 1996). Although, it is not clear if this effect is peculiar to the vectors tested by Hartikka *et al* or whether it is dependent upon the position of the resistance gene within the vector, it is noteworthy that the pBabe/*puro* vector encodes the

ampicillin resistance gene. By the same token, the pBabe/*puro* vector contains the puromycin resistance gene but pVR1255 does not encode any marker for selection in eukaryotic cells.

Insertion of the GPCR gene into pVR1255 was considerably less time-consuming than with pBabe/*puro*. Several attempts were also made to insert the GPCR into the pEGFP-N1 expression vector (Clontech) to generate a GPCR-GFP fusion but this was not achieved in the course of this project. The positive pVR1255 clones were still restricted in growth, yet large-scale plasmid preparations appeared to yield higher levels of DNA. Nonetheless, the transfection efficiency of 293 cells with pVR1255-GPCRHA3 was much reduced compared with a positive control plasmid expressing GFP and there appeared to be fewer viable cells post-transfection, thus indicating that pVR1255 only partially overcame the toxicity problem. The low number of cells that stained positively with the anti-haemagglutinin antibody suggests that there was negative selection of cells expressing high levels of the GPCR. It is not unusual for transforming transmembrane proteins to be toxic when overexpressed in eukaryotic cells. In addition to the KSHV GPCR, which has already been discussed, overexpression of the EBV LMP-1 gene has been shown to be toxic to mammalian cells (Hammerschmidt *et al.*, 1989).

The anti-haemagglutinin antibody reproducibly detected expression of the GPCR protein in patches consistent with localisation to the cell surface. The individual image slices generated using the confocal microscope (see 3.3.5B) indicate that the FITC signal is not co-incident with the nuclear propidium iodide staining. Although cell surface expression is likely for a transmembrane signalling protein, it would be necessary to confirm association with the plasma membrane by co-localisation with a cell surface marker such as MHC class I. The punctate expression pattern is typical of transmembrane proteins that are activated by aggregation such as EBV LMP-1 (Liebowitz *et al.*, 1986) and the epidermal growth factor receptor (Schechter *et al.*, 1979; Schreiber *et al.*, 1983).

4.3.3. Binding of IL-8 to the GPCR

The potential interaction of the GPCR with the chemokine, IL-8, was investigated using a radioactive binding assay. Chemokine binding has been demonstrated for three viral GPCRs so far. Despite sharing greater sequence identity with the CCR1 than CXCR2, the HVS GPCR exhibits a binding profile similar to CXCR2 (Ahuja & Murphy, 1993). The HCMV US28 protein binds CC chemokines such as RANTES (Vieira *et al.*, 1998) while the KSHV GPCR is a promiscuous receptor that interacts with both CC and CXC chemokines (Arvanitakis *et al.*, 1997). Since both gammaherpesvirus GPCRs bound CXC chemokines and the KSHV GPCR had highest affinity for IL-8, IL-8 appeared a logical first choice to test the binding activity of the GPCR.

Multiple attempts were made to detect binding of radiolabelled IL-8 to the GPCR1 3T3 cell clone or transiently transfected 293 cells but no difference was ever observed between the test samples and the negative controls. It is likely that low expression of the GPCR was a fundamental problem in this assay. Expression of the GPCR transcript had been verified in the GPCR1 cells but without an antibody, the presence of the protein could not be confirmed. There is little doubt that this cell clone expresses the GPCR protein as it exhibits transformed characteristics, however, the level of expression required to elicit transformation is likely to be far less than that required for ligand-binding or immunological analysis. A stable cell line expressing the pVR1255-GPCRHA3 construct may have provided the necessary substrate but this could not be generated, as the vector encodes no mammalian selection marker. It may have been possible to engineer a selectable marker into pVR1255 but the experiments of Hartikka *et al* have illustrated that the addition or replacement of DNA elements can have a profound effect on protein expression. It is also uncertain whether a stable cell line could support high-level expression of the GPCR or if it would simply result in selection of non-expressing cells. An inducible system allowing transient expression of the GPCR may have partially overcome the toxicity problem, at least in mammalian cells if not bacteria.

Ultimately, binding of radiolabelled IL-8 to positive control U937 cells demonstrated that the experiment itself was not flawed. As this is a relatively sensitive assay that should detect even low levels of bound ligand, an alternative explanation is that the MHV-68 GPCR does not have a high affinity for IL-8. Although IL-8 binding has been established for the KSHV and HVS receptors, MHV-68 has evolved to persist in rodents, which do not synthesise IL-8. It would therefore be interesting to repeat this experiment with a murine chemokine such as KC.

4.4. Transforming Activity of the GPCR

4.4.1. Focus Formation and Anchorage Independent Growth

The transforming activity of the GPCR was demonstrated using classical transformation assays. Transient transfection of NIH3T3 cells with transformation constructs (see diagram 3.4.1) resulted in moderate loss of contact inhibition and occasional focus formation. Due to the poor transfection efficiency of NIH3T3 cells, changes in growth characteristics only became obvious following selection of cells that had taken up the plasmid. Clones of puromycin resistant cells readily exhibited loss of contact inhibition and focus formation when maintained in a confluent monolayer for a period of 3-4 weeks. Transformed focal cell clones also displayed anchorage independent growth in soft agar, in contrast to cells stably transformed with the empty vector. Interestingly, independent cell clones carrying the same plasmid exhibited subtle variations in transformation characteristics, with some clones appearing to be more transformed than others. For instance, the two $GPCR^+$ cell lines, GPCR1 and GPCR2, both exhibited focus formation yet GPCR1 formed a greater number of foci than GPCR2. In the anchorage independence assay, GPCR1 and GPCR2 generated a similar number of colonies in soft agar but the morphology of the colonies differed: the colonies produced by GPCR1 were typically larger and appeared denser than the GPCR2 colonies. A similar distinction was also observed between LMP1 and LMP4, with LMP4 forming noticeably more colonies than LMP1. This may reflect a difference in the number of copies of the plasmid and/or the site of integration, which could affect the levels of protein expression and hence the growth properties of the cells. However, Southern analysis of the genomic DNA

would be required to confirm this. There were also qualitative differences in the cellular morphology of the transformed clones. Cells transformed with EBV LMP1 appeared smaller and rounder which may be a result of the known effect of this protein on the cell cytoskeleton (Panayiotides *et al.*, 1996). The number of colonies formed by the SV402 cells was low compared to the other cell clones. A likely explanation for this is that the pPVU-0 plasmid does not encode a mammalian selectable marker, which prevented selection of transfected cells. Although SV402 was propagated from a cellular focus, the small number of cells that stained positively for large T antigen suggests either that this cell strain was not clonal or that the plasmid had been selectively lost.

4.4.2. Subjectivity of Transformation Assays

It is not appropriate to compare the transforming activity of the GPCR with LMP and large TAg in the soft agar assay. As discussed previously (see section 1.9.1), changes associated with transformation are highly dependent on the method of selection, therefore evidence of one transformed characteristic does not presume possession of others (Smith *et al.*, 1971). In this case, the cell clones were selected on the basis of loss of contact inhibition and focus formation so it does not necessarily follow that they should display an equal ability to grow in soft agar. Likewise an ability to grow in low concentrations of serum could not be demonstrated for any of the transformed cell clones. Despite culturing the cells in a range of serum concentrations (0.1-5% NBC or FCS), conditions required for the survival of transformed cells and concomitant death of negative control cells could not be determined.

It is also fair to say that these cell culture-based assays are relatively subjective and results may vary in the hands of different experimenters. Not only is there variation between the strains of 3T3 cells used as the substrate in transformation assays but they also appear highly sensitive to changes in growth conditions. For instance, focus formation failed to occur when cells were maintained in 10% newborn calf serum as opposed to 10% foetal calf serum, presumably because growth factors present in the foetal calf serum were required for transformation.

4.4.3. Tumorigenicity in Nude Mice

The observation that transformed cells rarely exhibit a full repertoire of transformation-associated characteristics may also partially explain the failure of the transformed cell clones to produce tumours in nude mice. There is also an element of subjectivity in this experiment as the variation in the strain of nude mouse may have an effect. However, the inability of 3T3 clones expressing the SV40 large TAg to develop into tumours was surprising since others have previously demonstrated the tumorogenicity of this gene *in vivo* (Tooze, 1973). While Rat-1 cells transformed with EBV LMP-1 are tumorigenic in nude mice, the failure of transformed NIH3T3 cells to grow in nude mice has been previously described (Wang *et al.*, 1985). Even so, the transient tumorogenesis observed in the second experiment is perhaps more suggestive of tumour regression than a failure of the cells to grow at all.

Nude mice carry an autosomal recessive mutation on chromosome 11 that prevents development of the thymus and also renders them hairless (Byrd, 1993; Nehls *et al.*, 1994). Being athymic, they are T cell deficient and therefore lack the T cell help required to mount an antibody response (Pantelouris, 1968). However, there is evidence that nude mice have elevated levels of natural killer (NK) cells, perhaps to compensate for the lymphocyte deficiency (Grzelak *et al.*, 1984; Su *et al.*, 1993). There are a large number of reports describing the down regulation of MHC class I on transformed cells and tumour cells, presumably as a mechanism of immune evasion (Elliott *et al.*, 1989; Goodenow *et al.*, 1985; Hammerling *et al.*, 1987; Tanaka *et al.*, 1985). Since NK cells recognise cells that fail to express MHC class I, it is possible that the transformed cells injected into the nude mice were highly susceptible to destruction by NK cells. Therefore it would be interesting, using an immunohistochemical technique, to compare the level of positive staining for MHC class I in the transformed cell clones and negative control cells. Variation in the levels and activity of NK cells between different strains of nude mice may also account for the lack of reproducibility in these experiments (Romijn, 1985). The problem of elevated NK cell activity may be overcome by treating nude mice with an immunosuppressive drug such as pristane, which has a known affect on NK activity (Freund & Blair, 1982; Kripke & Weiss, 1970).

Conventional transformation assays were first developed in the 1950s and 1960s to analyse the biological effects of DNA tumour viruses. Although still very much used today, they are gradually being replaced or at least complemented by immunohistochemical assays that can detect transformation-associated changes in the cytoskeleton and the presence of tumour antigens.

4.5. Recombinant Virus

4.5.1. Homologous Recombination

Prior to the advent of molecular cloning techniques, which facilitated targeting, mutant viruses were isolated on the basis of altered plaque phenotype (Ejercito *et al.*, 1968), drug resistance (Tognon *et al.*, 1988) or host-range properties (Aurelian, 1964). Homologous recombination has been used to successfully target a wide range of herpesvirus genes, thus allowing gene function to be explored in the context of the whole virus. In particular, the generation of herpes simplex mutant viruses has provided a wealth of information concerning the roles of individual genes (Balan *et al.*, 1994; Forrester *et al.*, 1992). Targeted disruption of betaherpesvirus genes has also been described, including the MCMV and RCMV GPCRs (Beisser *et al.*, 1998; DavisPoynter *et al.*, 1997). Homologous recombination in gammaherpesviruses appears to present a greater challenge than in alphaherpesviruses and betaherpesviruses, nevertheless, several recombinant viruses with dysfunctional genes have been described (Borza & Hutt-Fletcher, 1998; Marchini *et al.*, 1992; Wang & Hutt-Fletcher, 1998). The inability of EBV and KSHV to grow permissively in tissue culture has hampered the study of these viruses at the molecular level. Conversely, the relatively efficient replication of MHV-68 *in vitro* indicates that it would be a more amenable virus for genetic manipulation and the subsequent analysis of recombinant viruses *in vivo*. Recombinant MHV-68 viruses generated to date include "knock-outs" of the M1 and cyclin D genes (Clambey *et al.*, 2000; Hoge *et al.*, 2000; Simas *et al.*, 1998; van Dyk *et al.*, 2000).

The homologous recombination strategy relies upon the insertional mutagenesis or complete replacement of a gene with a selectable marker that can be used to identify

the recombinant. A number of different selectable markers have been used including the *E.coli* guanosine phosphoribosyl transferase (*gpt*) gene (Greaves *et al.*, 1995), the hygromycin phosphotransferase gene (Marchini *et al.*, 1992), the HSV-1 thymidine kinase gene (Post *et al.*, 1981), the neomycin resistance gene (Grassmann & Fleckenstein, 1989; Wang & Hutt-Fletcher, 1998), the β-galactosidase gene (van Dyk *et al.*, 2000) and GFP (Vieira *et al.*, 1998). GFP is a powerful non-evasive marker, which offers the advantage of detecting "tagged" virus in individual infected cells. However, as is discussed below, GFP may not have provided sufficient selection criteria for purification of recombinant MHV-68 and that using a construct with a combination of selectable markers might improve the chance of isolating a pure homologous recombinant.

4.5.2. RCVI

The MHV-68 GPCR was targeted using a recombination construct encoding approximately 2kb homologous flanking regions on either side of the GFP gene. The construct recombined with the MHV-68 genome resulting in recombinant virus that was selected on the basis of GFP expression. Successive rounds of plaque purification and limiting dilution were used to purify the recombinant virus to a standard where no wild type GFP^- plaques were visible. However, Southern analysis revealed that wild type virus was still present in the sample and that illegitimate recombination of the construct had occurred. The small graduations or laddering effect detected with two of the Southern probes suggested that recombination had taken place adjacently to a region of repetitive DNA. Detailed analysis of the banding pattern revealed partial deletion and integration of the construct at the left-hand end of the genome.

This discovery raised several issues. Firstly, non-homologous recombination in this region of the genome had been observed previously using different recombination constructs (Roy, unpublished observation), implying the presence of a recombinational "hot-spot". The subsequent insertion of the construct at the correct site in MHV-76 (RCVII) shows that the homologous flanking regions in the recombination construct were of sufficient length to achieve specific integration.

Taken together, these findings indicate that integration at the left-hand end is not a completely random event. As has been proposed for HVS, this region of the MHV-68 genome is a potential site for the virus origin of replication (Bowden *et al.*, 1997; Grassmann & Fleckenstein, 1989). If so, this region may adopt a more open conformation, which would make it more accessible for recombination.

The second issue to arise from this experiment was the possibility that the GPCR might be an essential gene or at least confer a growth advantage on the virus. In the former scenario, deletion of ORF74 would not generate viable recombinant virus, whereas in the latter, the virus might be severely debilitated. If the GPCR were an essential gene, a "knock-out" virus might still be able to replicate in the presence of wild type virus if the mutation could be complemented in trans. This might explain the persistence of wild type virus through numerous rounds of purification. In either case, selection of GFP^+ virus using plaque purification and limiting dilution would favour the selection of illegitimate recombinants that were not growth inhibited. Thirdly, the time spent attempting to purify this non-homologous recombinant obviated the need for a PCR-based assay to determine whether homologous recombination had occurred following the initial transfection.

4.5.3. RCVII

To try and circumvent the problem of illegitimate recombination, targeted deletion of the GPCR was attempted in a different virus. MHV-76 was isolated concurrently with MHV-68 (Blaskovic *et al.*, 1980) and limited sequence analysis has only revealed one major difference between the two viruses: the absence of genes M1-M4 and the tRNAs (Macrae, unpublished), all of which are encoded at the left-hand end of the MHV-68 genome. It was therefore reasoned that MHV-76 might also lack the highly recombinogenic region encoded by MHV-68. However, the subsequent generation of a left-hand end recombinant virus using MHV-76 (RCVII) as a target virus disputes this. If anything, it strengthens the evidence of similarity between MHV-76 and MHV-68 and suggests that the hot-spot is likely to be situated within the terminal repeats rather than in the unique region of the genome.

The PCR assay designed to identify GPCR-negative virus provided the first indication that homologous recombination had occurred, and this was later confirmed by Southern analysis. It was possible using PCR to screen large numbers of viral isolates and the sensitivity of the technique facilitated detection of potentially rare recombinant virus. If this assay had been developed previously it may well have detected the presence of the homologous recombinant in the first generation MHV-68 recombinant virus. Following the first round of plaque purification, the ratio of recombinant to wild type virus was estimated, revealing that wild type virus was predominant. Using the sample with the highest ratio of recombinant to wild type virus, enrichment of GFP^+ virus was achieved by plaque purification. Following each round of purification, virus isolates were tested for the presence of the homologous recombinant using PCR. It was observed that the number of positive samples did not increase with successive rounds of purification, despite the absence of visible wild type plaques. Preliminary Southern analysis (not shown) indicated the faint presence of the homologous recombinant but also revealed a high level of illegitimate recombinant. Unfortunately, the PCR assay failed to distinguish between wild type MHV-76 and virus that had recombined in the left-hand end. Potentially, there were four viruses present in the population: wild type, left-hand end recombinant, homologous recombinant and virus containing insertions at both loci.

4.5.4. Purification Strategies

The inability to purify the homologous recombinant or at least enrich its presence in the population was puzzling. Plaque purification is an established method for isolating recombinant viruses. The selection of single, well-isolated plaques that have arisen from a single plaque-forming unit will, in most cases, eventually yield pure virus. The persistence of wild type virus or left-hand end recombinant, which grows essentially like wild type virus *in vitro*, again raised the possibility that the homologous recombinant was a replication deficient virus that could not be propagated without the support of wild type virus.

To address this, plaque purification was carried out using 3T3 cells expressing the GPCR (GPCR1) to determine whether recombinant GPCR could complement the

potentially debilitating mutation and thus facilitate the growth of plaques containing pure virus. Although previous experiments had suggested that the GPCR1 cells do not express the GPCR at a high level, it was reasoned that since the GPCR transcripts are at such low abundance during lytic infection, there should be sufficient expression to complement the virus. Regrettably, it was found that the virus did not infect these cells efficiently and that all the plaques tested by PCR were contaminated with wild type or left-hand end recombinant virus. It may have been advisable to persevere with this strategy and produce a complementary cell line capable of generating a large number of plaques to increase the chance of finding a pure virus clone. However, this problem presented itself at a very late stage in the project and there was no guarantee that a different $GPCR^+$ cell line would be any more successful. The other theory was that deletion of the GPCR had had a deleterious affect on the transcription of other genes in the same locus. Although care was taken to avoid disruption of the ORF73 and ORF75 coding regions and promoters, it is still possible that the GPCR coding region contains long-range elements that regulate their expression. In addition, the insertion of the CMV promoter immediately upstream of ORF73 could have had potentially adverse effects on its transcription. ORF73 is a potential homologue of KSHV LANA and EBV EBNA1 and may therefore have an essential transactivation function. However, it would be impossible to establish if ORF73 transcription has been affected without actually purifying the recombinant virus.

An alternative strategy that avoided the necessity for lytic replication was to infect B cells and reactivate latent virus. It was acknowledged, however, that this strategy would only be successful if the recombinant virus were at a growth disadvantage rather than completely replication deficient. As previously discussed, there is some evidence that B cells support only one episomal variant of EBV and are resistant to superinfection (Kintner & Sugden, 1981; Reisman & Sugden, 1984), which might allow reactivation of pure virus from individually cloned B cells. Unfortunately, the expression of GFP proved to be unstable in the virus-infected NS0 cells, indicating that transcription of the GFP gene may have been suppressed during virus latency. This is supported by the observation that the addition of phorbol ester (TPA)

noticeably increased the number of GFP^+ cells. Physical isolation of individual B cells that were transiently expressing GFP was attempted using suction with a micropipette but without success. The inability to clone a single GFP^+ B cell meant that it would be necessary to screen a very large number of B cells in the population to find one that was infected with the recombinant. Approximately thirty plaques generated from reactivated B cells were tested using PCR and all contained the wild type or left-hand end recombinant virus, which disputes the original hypothesis that a single episomal variant predominates in latently infected B cells. Despite evidence that pure virus can be reactivated from the latent B cells of infected mice, a similar result was obtained following infection of mice with the mixed virus population. Curiously, the presence of both recombinant and wild type/left-hand end recombinant virus was evident in all the plaques screened. This may reflect the persistence of infectious virus in the spleen at this time-point but again casts doubt on the theory that B cells generally harbour only one latent virus genotype.

4.5.5. Different Approaches to Generating a $GPCR^-$ Mutant

In conclusion, the failure to purify a recombinant virus lacking the GPCR using a variety of strategies strongly indicates that replication of this virus was severely debilitated if not unviable. With the benefit of hindsight, this goal might have been approached differently with greater success. Firstly, using GFP as the sole marker of recombinant virus did not allow differentiation between homologous and non-specific recombination of the construct. There was also no facility for negative selection of wild type virus. It might be possible to distinguish illegitimate recombinants using a construct encoding a selectable marker such as blue fluorescent protein outside the homologous flanking regions. However, as shown with RCVI, non-specific integration was accompanied by partial deletion of the construct, which would create the potential for false positives using this option. Secondly, removal of the whole GPCR coding region now seems unnecessary. Although retention of signalling function has been demonstrated for a GPCR encoding only five transmembrane regions (Ling *et al.*, 1999), destroying the region involved in G protein coupling should be sufficient to inactivate the GPCR. Restricting the level of

disruption might assuage the potential impact on the expression of genes surrounding the GPCR.

There are of course alternative strategies for the generation of mutant viruses that avoid the purification of recombinant from wild type virus. One approach would be to use a cosmid system (Cunningham & Davison, 1993; de Wind *et al.*, 1990; van Zijl *et al.*, 1988). The advantage of using a cosmid library is that it allows mutation of the gene of interest and cloning of the modified cosmid within bacteria. Restriction analysis or limited sequencing could be used to identify clones carrying the mutation. Providing any overlapping sequences encoding regions of the gene of interest are excised from other cosmids, the intact mutant virus genome can be reassembled by recombination of the individual genomic fragments in mammalian cells. The other attractive feature of this method is that it does not require insertion of a selectable marker thus allowing the generation of more discrete mutations within the gene of interest. The only drawback is that repetitive sequences such as herpesvirus terminal and internal repeats tend to be unstable in bacteria (Gray & Kaerner, 1984; Quinn & McGeoch, 1985; Weller *et al.*, 1985).

The bacterial artificial chromosome (BAC) system has also attracted a great of interest in terms of generating herpesvirus recombinants. Entire virus genomes have been inserted into single BACs (Delecluse *et al.*, 1998; Horsburgh *et al.*, 1999; Messerle *et al.*, 1997), thus allowing mutation of viral genes in bacteria. Production of an MHV-68 BAC has been reported which may provide a useful way of generating MHV-68 recombinants (Adler *et al.*, 2000), although the stability of virus genomes within BACs has been called into question.

4.5.6. Conclusions and Potential Functions of the GPCR

This work represents an initial characterisation of the MHV-68 GPCR. Transcriptional analysis detected expression of the GPCR at early and late time-points during lytic infection on multiple rare polycistronic transcripts. Co-expression of the GPCR with v-Bcl-2 has also been demonstrated *in vitro*. *In vivo*, the evidence suggests that the GPCR and v-Bcl-2 are co-expressed and may be latency-associated.

Evidence has also been presented that the GPCR protein is expressed on the cell surface and that its overexpression is toxic to both prokaryotic and eukaryotic cells. Classical transformation assays have established a transforming activity mediated by the GPCR, which indicates that it is a viral oncogene with a variety of potential functions in viral pathogenesis.

An immunomodulatory role has been postulated for the MHV-68 GPCR. As previously discussed, several poxvirus cytokine receptor homologues appear to down regulate the inflammatory response by sequestering host cytokines. In addition, expression of the HCMV US28 GPCR *in vitro* leads to a decrease in the concentration of RANTES in the medium (Vieira *et al.*, 1998). However, attempts to express the MHV-68 GPCR *in vitro* and the barely detectable level of transcription during productive infection, suggest that it would be ineffectual in dampening the immune response. Instead, the low level of expression and the transforming activity of the GPCR are more consistent with a signalling function. The competitive binding assay did not demonstrate an affinity of the MHV-68 GPCR for IL-8, however, it is possible that this receptor may mediate signalling or immunomodulatory activities via other cytokines.

The KSHV GPCR is a functional, constitutively active receptor that exhibits transforming activity and induces the angiogenic growth factor, VEGF (Arvanitakis *et al.*, 1997; Bais *et al.*, 1998). The transforming activity of the MHV-68 GPCR suggests that it too is a viral oncogene, which may contribute to lymphomagenesis directly, or indirectly by activating other genes involved in transformation. The involvement of the KSHV GPCR in tumorigenesis has been questioned since it is a tightly regulated lytic gene, not expressed during latency. However, expression of the KSHV GPCR has been demonstrated in KS lesions in the small proportion of cells undergoing productive infection. A paracrine mechanism of transformation has therefore been proposed which would be consistent with the evidence of cytokine-mediated proliferation in the pathogenesis of KS. The MHV-68 GPCR is also a lytic gene but possible latency-associated expression has been detected *in vivo*, which indicates a role for the GPCR in virus persistence. In particular, the co-expression of

the GPCR with v-Bcl-2 suggests that these genes may act synergistically to stimulate cell proliferation or reactivate productive infection without inducing apoptosis. It is perhaps not accidental that three MHV-68 cellular homologues of genes involved in cell proliferation and survival are clustered together in the genome. It is possible that v-cyclin, v-Bcl-2 and v-GPCR are expressed as a growth-promoting cassette with oncogenic potential.

As previously discussed, there seems no apparent advantage to the virus in stimulating tumorigenesis, which may result in the demise of the host. In support of this, herpesviruses very rarely cause tumours in their natural immunocompetent hosts. However, the sheer number of GPCRs encoded by herpesviruses indicates that they must confer a selective advantage upon the virus. A proliferative receptor could be valuable to the virus in several different ways. For instance, the MHV-68 GPCR may provide a proliferation signal during acute infection, which would serve to increase the numbers of infected cells. A more likely explanation is that the GPCR functions during chronic infection to promote persistence of the virus. It may be that the GPCR activates cell division in latently infected cells as a way of increasing the number of cells harbouring the virus. Alternatively, the GPCR may initiate productive replication, which would generate a far greater number of infected cells. This would be consistent with the low level of productive infection detected in the lungs of persistently infected mice (Stewart *et al.*, 1998). Although there is no direct evidence for a role in reactivation, induction of productive replication by phorbol esters is based on their structural similarity to diaglycerol, which activates protein kinase C. It is interesting that the KSHV GPCR and GPCRs in general have been shown to transduce signals via this pathway.

Another potential role for the GPCR might involve B cell trafficking. EBV induces a cellular GPCR, CCR7, which appears to be involved in homing of B cells to specific subatomic compartments of the spleen. In transgenic mice lacking CCR7, activated B cells failed to migrate from the T cell rich zone into the B cell rich zones of the splenic follicles and germinal centres were not formed (Forster *et al.*, 1996). The MHV-68 GPCR could possibly function to increase the number of infected B cells

by targeting them to germinal centres of the spleen for expansion. Evidence for a role in cellular tropism is provided by recombinant RCMV lacking the GPCR, R33. This virus spreads successfully to the salivary glands of infected rats but once there, fails to replicate, indicating a cell-type specific function (Beisser *et al.*, 1998).

In conclusion, the MHV-68 is a viral oncogene, which may contribute to the pathogenesis of MHV-68 by several different routes. While the evidence favours a proliferative signalling function that promotes MHV-68 persistence, it is not incompatible with other growth-promoting or trafficking roles in acute infection.

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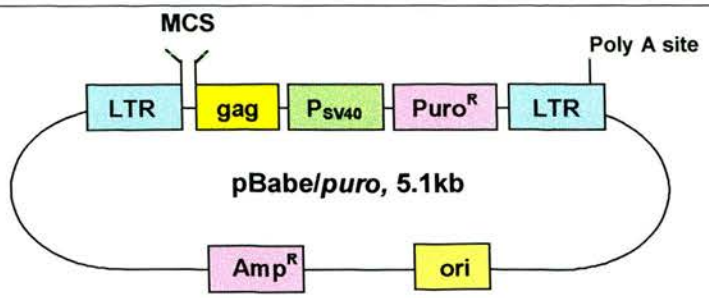
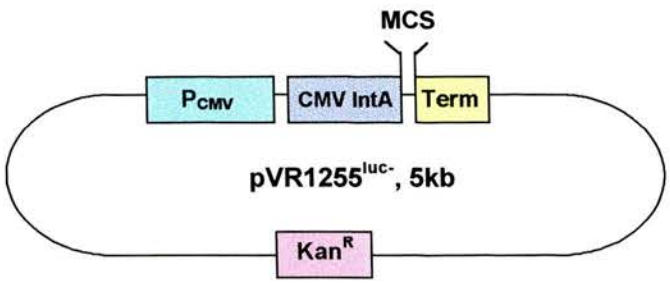
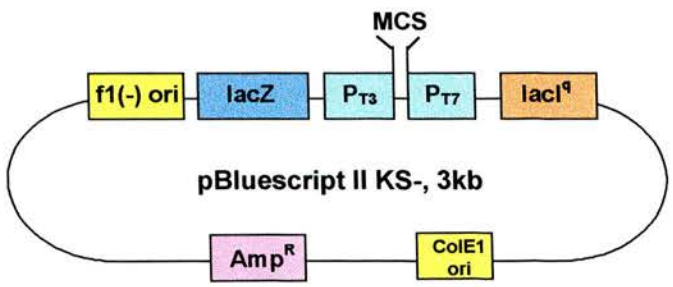
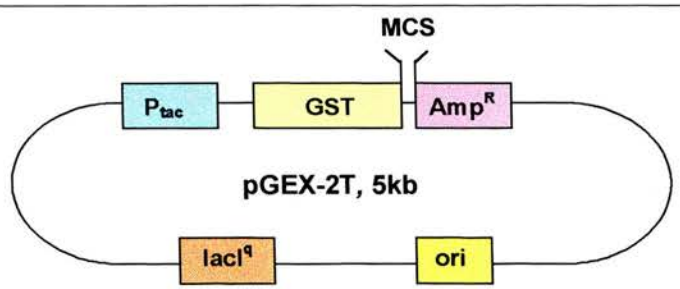
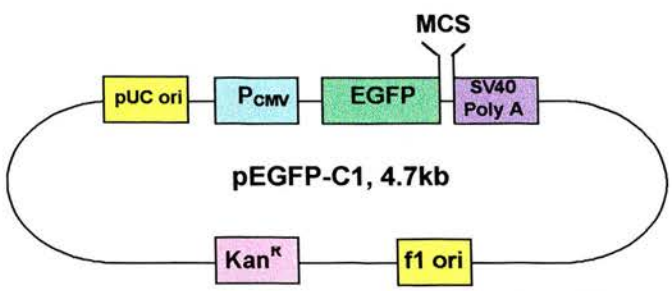
Appendix 1

SENSE (S) ANTISENSE (A)	PRIMER PAIR	ANNEALING TEMPERATURE	AMPLIFIED REGION
	*Bam*HI		
1S	5'-dGCGCGGATCCGCCACGATGCTTGTCCTGCG-3'		MHV-68 105057-106070
	*Eco*RI	59°C	ORF74
1A	5'-dGCGCGAATTCTTAGGAGCTTAGTCTACAAACTG-3'		Product size: 1030bp
	*Bam*HI		
2S	5'-dGCGCGGATCCGAAGATCTTCAGGCATTCTTAG-3'		MHV-68 105084-105177
	*Eco*RI	53°C	ORF74 N-terminus
2A	5'-dGCGCGAATTCTACAGGGGCTCGCCAAATCT-3'		Product size: 1013bp
	*Bam*HI		
3S	5'-dGCGCGGATCCGCCACGATGCTTGTCCTGCG-3'		MHV-68 105057-106067
	*Eco*RI STOP HA Epitope	50°C	HA-tagged ORF74
3A	5'-dGCGCGAATTCTTA AGCGTAGTCTGGAACGTCGTATGGGTA		Product size: 1060bp
	TGGGTAGGAGCTTAGTCTACAAA-3'		
	*Bam*HI		
4S	5'-dGCGCGGATCCGCCACGATGCTTGTCCTGCG-3'		
	*Eco*RI STOP Triple HA Epitope		MHV-68 105057-106067
4A	5'-dGCGCGAATTCTTAAGCGTAGTCTGGAACGTCGTATGGGTA	50°C	3xHA-tagged ORF74
	AGCGTAGTCTGGAACGTCGTATGGGTA		Product size: 1111bp
	AGCGTAGTCTGGAACGTCGTATGGGTA		
	TGGGTAGGAGCTTAGTCTACAAA-3'		
	*Bam*HI		
5S	5'-dGCGCGGATCCATGAGTCATAAGAAAAGCGGGA-3'		MHV-68 103418-106067
	*Eco*RI	59°C	M11→ORF74
5A	5'-dGCGCGAATTCTTAGGAGCTTAGTCTACAAACTG-3'		Product size: 2694bp
6S	5'-dTTAGAAGGCACTATGACAGC-3'		MHV-68 103560-103779
		51°C	vBcl-2
6A	5'-dTGTGTCATGCAATCGTTCAA-3'		Product size: 219bp
	*Xho*I		MHV-68 103171-105064
7S	5'-dGCGCCGATCGCCGAGTTCATAGTGACAG-3'		Left flank
	*Eco*RI	57°C	Recombinant virus
7A	5'-dGCGCGAATTCGCTCCTAATCTCTAGATG-3'		Product size: 1913bp
	*Eco*RI		MHV-68 106063-108018
8S	5'-dGCGCGAATTCGCTCCTAATCTCTAGATG-3'		Right flank
	*Pvu*I	47°C	Recombinant virus
8A	5'-dGCGCCGATCGCCGAGTTCATAGTGACAG-3'		Product size: 1975bp
9S	5'-dGCGCCTGCGATAGATCATCTGTCTG-3'		Recombinant MHV-76
		57°C	
9A	5'-dGCGCGGGAACATACGTCATTATTGAC-3'		Product size: 2301bp
10S	5'-dGCGCCTGCGATAGATCATCTGTCTG-3'		WT MHV-76
		57°C	103102-106067
10A	5'-dGCGCGAATTCTTAGGAGCTTAGTCTACAAACTG-3'		Product size: 2965bp

Appendix 1 Contd.

11S	*Hind*III 5'-dCGCGAAGCTTAAAACCCTCAACATGTGTGG-3'	55°C	MHV-68 gp150
11A	*Eco*RI 5'-dGCGCGAATTCCAGGTCCAAATCAAGACAC-3'		Product size: 1560bp
12S	5'-dGCGCCGTCATAGGACAATGATGAG-3'	53°C	5'RACE
12A	5'-dGCGCCAATGCTACCTGGATTCCAG-3'		1st round PCR
13 S	5'-dGCGCCTGAAGATCTTCCACGTC-3'	49°C	5'RACE
13A	5'-dGCGCGTCTGTGCTTGGAAATCTG-3'		2nd round PCR
14A	5'-GCCAACATGCACAC-3'	42°C	5'RACE Reverse Transcription Primer
15A	$p(dN)_6$	42°C	Random Hexamer RT-PCR
16A	*Eco*RI 5'-dGAGCTCGAATTCCAGCTG$(T)_{15}$-3'	42°C	Oligodeoxythymidine RT-PCR
17S	*Eco*RI 5'-dGCGCGAATTCGTTCATAGCCCATATATGGAG-3'	55°C	pEGFP-C1 35-1645 pCMV + GFP coding region
17A	*Eco*RI 5'-dGCGCGAATTCCGCGTTAAGATACATTGATGAG-3'		Product size: 1571bp
18S	*Nhe*I 5'dCGCGGCTAGCTGCCCTGAGGATGGAACACGACCTTGAG-3'	53°C	EBV LMP-1
18A	*Xho*I 5'dCGCGCTCGAGTTAGTCATAGTAGCTTAGCT-3'		Product size: 1.2kb
19S	5'-dTGTGATGGTGGGAATGGGTCA-3'	55°C	Mouse β-actin
19A	5'-dTTTGATGTCACGCACGATTTC-3'		Product size: 514bp

Appendix 2

	(Morgenstern & Land, 1990)
	(Hartikka *et al.*, 1996) Obtained under license from Vical Inc.
	Stratagene
	Amersham Pharmacia
	Clontech

Appendix 3

ANTIBODY	CONCENTRATION	SUPPLIER
Anti-haemagglutinin high affinity rat monoclonal antibody (Clone 3F10)	1:1000 - IF 1:6000 - Western	Roche
Anti-SV40 large T antigen monoclonal antibody (Clone OG5)	1:1000 - IF	TCS Biologicals
Anti-GST polyclonal antibody	1:500 - Western	Stewart, unpublished
Anti-MHV-68 polyclonal antibody	1:500 - Western	(Sunil-Chandra *et al.*, 1992a)
Alkaline phosphatase conjugated swine anti-rabbit polyclonal antibody	1:1000 - Western	Dako
Biotin-labelled donkey anti-sheep polyclonal antibody	1:10,000 - Western	Sigma
FITC-labelled goat anti-rat polyclonal antibody	1:250 - IF	Pharmingen
Biotin-labelled rabbit anti-rat polyclonal antibody	1:10,000 - Western 1:300 - IF	Dako
Alkaline phosphatase conjugated streptavidin	1:1000 - Western 1:500 - IF	Roche

IF = Immunofluorescence

Appendix 4

Amersham Pharmacia Biotech UK
Amersham Place, Little Chalfont
Buckinghamshire HP7 9NA
UK

Bantin & Kingman Ltd.
Hull
UK

BD PharMingen
10975 Torreyana Road
San Diego, CA 92121
USA

BDH Merck Ltd.
Hunter Boulevard, Magna Park
Lutterworth LE17 4XN
UK

Beckman Coulter, Inc.
4300 N. Harbor Boulevard, P.O. Box 3100
Fullerton, CA 92834-3100
USA

BIO-RAD Laboratories
2000 Alfred Nobel Drive
Hercules, CA 94547
USA

Cecil Instruments Ltd.
Milton Technical Centre
Cambridge CB4 6AZ
UK

Clontech Laboratories Inc.
1020 East Meadows Circle
Palo Alto, CA 94303-4230
USA

Cruachem Ltd.
West of Scotland Science Park
Acre Road
Glasgow, G20 OUA
UK

DAKO A/S
Produktionsvej 42
DK-2600, Glostrup
Denmark

DuPont NEN® Research Products
549 Albany Street
Boston, MA 02118
USA

EquiBio
The Wheelwrights
Boughton Monchelsea,
KENT, ME17 4LT, UK

Flowgen
Novara House, Excelsior Road
Ashby Park, Ashby de la Zouch
Leicestershire LE65 1NG
UK

Hybaid Limited
Action Court, Ashford Road
Ashford, Middlesex, TW15 1XB
UK

Leitz Tooling UK Ltd.
Harlow
UK

Life Technologies Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
UK

Millipore (U.K.) Ltd.
The Boulevard, Blackmoor Lane
Watford, Hertfordshire WD1 8YW
UK

Molecular Dynamics
928 East Arques Avenue
Sunnyvale, CA 94086-4520
USA

Nalge Nunc International
75 Panorama Creek Drive
Rochester, NY 14625
USA

National Diagnostics
Fleet Business Park
Itlings Lane, Hessle
Hull HU13 9LX, UK

Nikon Corporation
Fuji Building
Marunouchi, Chiyoda-ku
Tokyo 100
Japan

Promega Corporation
Madison, WI 53711-5399
USA

QIAGEN Ltd.
Boundary Court, Gatwick Road
Crawley, West Sussex RH10 2AX
UK

Roche Diagnostics Ltd.
Bell Lane, Lewes
East Sussex BN7 1LG
UK

Shandon Scientific Ltd.
Chadwick Road, Astmoor
Runcorn, Cheshire WA7 1PR
UK

Sigma-Aldrich Co. Ltd.
Fancy Road, Poole
Dorset BH12 4OH
UK

Stratagene Cloning Systems
11011 North Torrey Pines Road
La Jolla, CA 92037
USA

Surgipath Europe , Ltd.
Venture Park, Stirling Way
Bretton, Peterborough
PE3 8YD, UK

Takara Biomedical Europe S.A.
Europarc des Barbanniers
6 Place du Village,
92230 Gennevilliers
France

UVP Inc.
2066 W. 11th Street
Upland, CA USA 91786
USA

Vical Incorporated
9373 Towne Centre Drive, Suite 100
San Diego, CA 92121-3088
USA

Wallac
Perkin Elmer House
204 Cambridge Science Park
Milton Road
Cambridge, UK

Whatman International Ltd.
St. Leonards Road
20/20 Maidstone
Kent ME16 OLS, UK